REGULATION AND DYNAMICS OF THE MSS4 PHOSPHOINOSITIDE KINASE COMPLEX

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REGULATION AND DYNAMICS OF THE MSS4 PHOSPHOINOSITIDE KINASE COMPLEX

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Phosphatidylinositol-4,5-bisphosphate, $PI(4,5)P_2$, is an essential signaling lipid that regulates key processes such as endocytosis, exocytosis, actin cytoskeletal organization and calcium signaling. Maintaining proper levels of $PI(4,5)P_2$ at the plasma membrane is crucial for cell survival and growth. I have characterized the conserved PI4P 5-kinase, Mss4, in budding yeast cells. Mss4 forms dynamic, oligomeric structures at the plasma membrane that are termed PIK patches. The dynamic assembly and disassembly of Mss4 PIK patches may provide a mechanism to precisely modulate Mss4 kinase activity, as needed, for localized regulation of $PI(4,5)P_2$ synthesis. Furthermore, I identify a tandem PH domain-containing protein, Opy1, as a novel Mss4-interacting protein that partially co-localizes with PIK patches. Based upon genetic, cell biological, and biochemical data, I propose that Opy1 functions as a co-incidence detector of the Mss4 PI4P 5-kinase and $PI(4,5)P_2$ and serves as a negative regulator of $PI(4,5)P_2$ synthesis at the plasma membrane. My results also suggest that additional conserved tandem PH domain-containing proteins may play important roles in regulating phosphoinositide signaling.

BIOGRAPHICAL SKETCH of YADING LING

Yading was born and raised in a small town called Pingwang (which means ‘peace and hope’) in eastern China. He attended Suzhou High School, in Jiangsu province in 1998. Yading became interested in chemistry in high school, so he attended Peking University to study material chemistry in 2001. During college, Yading spent two years studying ultrahigh-density data storage on new materials in Dr. Zhongfan Liu’s lab. He graduated from Peking University in 2005 and accepted an offer from the Department of Chemistry and Biochemistry at University of California, San Diego. After doing a series of rotations, Yading found that the research in Dr. Scott Emr’s lab was very interesting, so he joined the lab in 2006. One year later, after receiving his master’s degree at UCSD, Yading moved to Cornell University with the Emr lab and continued his research on the phosphoinositide kinase Mss4. He published his work on Mss4 recently in *The EMBO Journal*. Now Yading hopes he can apply what he has learned to solve some real world challenges in the coming future.

This work is dedicated to my parents, Jianying Gu and Xiaoyuan Ling.

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It's exciting, but also a bit difficult to believe that my Ph.D. life at Cornell will come to an end soon. It feels like it was yesterday that I joined the Emr lab and started working on a protein called Mss4. Looking backwards, the road to the Ph.D. is not easy, and full of ups and downs. I really have to thank a lot of people who brought me to this milestone in my life.

First of all I would like to thank my advisor, Scott Emr. Scott is a very supportive mentor and always helped me to keep my spirit up even when my project did not go so well. He is very insightful and focuses on the most important scientific questions, which was important for guiding the direction of my research. His breadth of knowledge and his passion towards science greatly inspired me in my graduate study.

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LIST OF ABBREVIATIONS

ARF, ADP ribosylation factors

CAPS, Ca^{2+}-dependent activator protein for secretion

DAG, diacylglycerol

DCV, dense-core vesicle

ER, endoplasmic reticulum

ERM, E for ezrin, R for radixin and M for moesin

FERM, F for 4.1 protein, E for ezrin, R for radixin and M for moesin

FYVE, F for Fab1, Y for YOTB, V for Vac1, and E for EEA1

GAP, GTPases-activating protein

GEF, Guanine nucleotide exchange factor

IP_3, inositol trisphosphate

MAPK, mitogen-activated protein kinase

NLS, nuclear localization signal

PA, phosphatidic acid

PH domain, pleckstrin homology domain

PIPKI, type I phosphoinositide kinase

PIP, phosphoinositide

PITP, phosphatidylinositol/phosphatidylcholine transfer protein

PKA, protein kinase A

PKC, protein kinase C

PLC, phosphalipase C

PLD, phosphalipase D

PM, plasma membrane

PtdCho, phosphatidylcholine

PtdIns, phosphatidylinositol

ROCK, Rho kinase

SILAC, Stable isotope labeling by amino acids in cell culture

SNARE, SNAP (Soluble NSF Attachment Protein) receptor

WASP, Wiskott–Aldrich Syndrome Protein

Chapter 1

Introduction

Phosphoinositides: essential signaling lipids in cellular membranes

Biological membranes are composed of lipid bilayers embedded with various trans-membrane proteins. They function as selective barriers and are essential for living cells. One unique feature that differentiates eukaryotic cells from prokaryotic cells is that membrane-bound organelles with distinct functions are compartmentalized in the cytoplasm. Compartmentalized organelles perform specific functions based on the enzymatic activities associated with them. For instance, the endoplasmic reticulum (ER) contains chaperones that fold newly synthesized proteins, while the lysosomes contain a variety of digestive enzymes that degrade proteins and lipids. These organelles are enclosed by distinct biological membranes in the cytoplasm. Dynamic exchange of membranes between different organelles takes place every second through vesicular transport reactions. To maintain organelle identity, the lipids in the membranes are rapidly metabolized by a set of local enzymes. Accordingly, cells precisely control the constituents of organelles by the correct targeting of proteins in coordination with the synthesis, transport and turnover of lipids.

Hundreds, if not thousands, of different lipid species are present in biological membranes, which can be classified into eight categories: fatty acyls, glycerol lipids, glycerolphospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides (Fahy et al, 2009). Glycerolphospholipids have important structural, energy storage and signaling functions. The phosphorylated derivatives of the lipid phosphatidylinositol (PtdIns), collectively known as phosphoinositides (PIP), are a major class of glycerolphospholipids that play very important roles in cells (Hokin, 1985). Phosphorylation and dephosphorylation can occur at the hydroxyl groups at positions D3, D4 and D5 on the inositol ring of PtdIns (Figure 1.1). Sequential phosphorylation events can modify PtdIns into a total of seven distinct PIP species, which can be inter-converted to each other by a set of PI kinases and PI phoshatases in the cell (Figure 1.2).

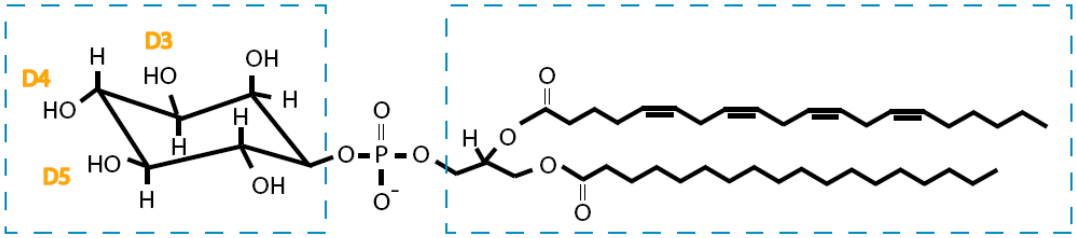
Each PIP has its unique sub-cellular distribution pattern, being abundant only at a subset of cellular membranes. Studies in the budding yeast *Saccharomyces cerevisiae* have shown that PI4P is enriched in the Golgi apparatus and the plasma membrane (PM), PI3P and PI(3,5)P_2 are mainly detected at the endosomal and lysosomal membranes respectively; while PI(4,5)P_2 is located predominantly at the PM (Figure 1.3). The restrictive localization of PIPs to specific membranes is a result of the highly regulated synthesis and turnover of lipids: the initial generation of PIPs at distinct cellular membranes is a consequence of the selective localization of PI kinases, while the maintenance of this unique PIP localization pattern is controlled by the PI

phosphatases that turn over mis-localized PIPs in cells. Importantly, studies in different organisms demonstrate that the compartment specific localization of PIPs is conserved (Behnia & Munro, 2005). Together with proteins such as the SNAREs and the small GTPases (e.g. Rabs and Arfs), PIPs serve as the lipid code to define organelle identity.

In addition, PIPs are important signaling molecules at cellular membranes. They serve as precursors of a variety of second messengers, such as DAG and IP_3 (Figure 1.2), and as signaling molecules themselves by recruiting and/or activating downstream effectors at cellular membranes (Figure 1.1B). Downstream effector proteins bind PIPs through a set of conserved modular domains, including the PH, PX, FYVE and C2 domains (Lemmon, 2003; Lemmon, 2008). PIPs often act in concert with other proteins (e.g. small GTPases), interacting with downstream effectors with high affinity and recruiting them to membranes to initiate various signaling pathways. This dual input system is often termed the 'coincidence detection' mechanism (Di Paolo & De Camilli, 2006). Moreover, PIPs provide both spatial and temporal signaling cues: (1) Each PIP is enriched at specific organelle membranes, thereby activating downstream signaling pathways at unique cellular locations; (2) PIPs are short-lived signaling molecules that can be turned over rapidly by PIP phosphatases.

Figure 1.1

A


D3
D4
D5
H
HO
OH
O
P
O
O-
H
O
inositol headgroup
phosphodiester link
diacylglycerol

B

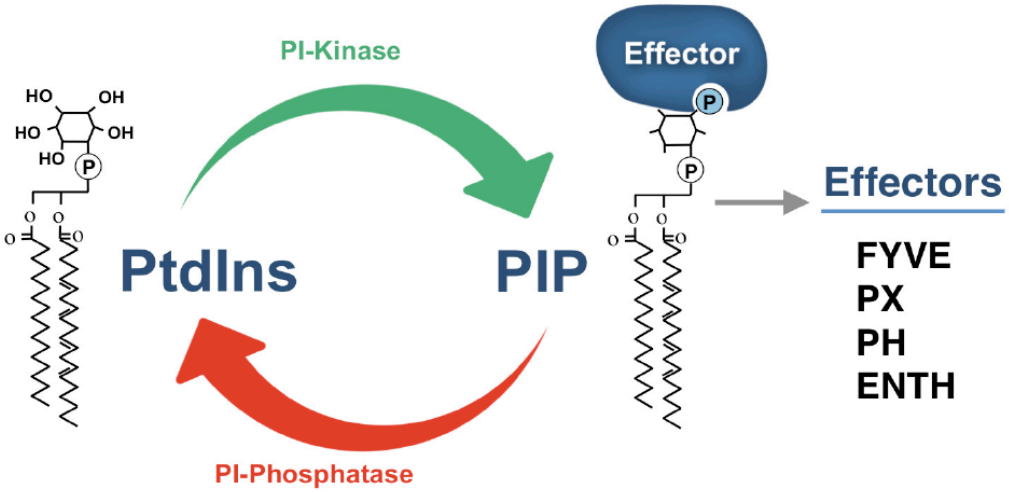
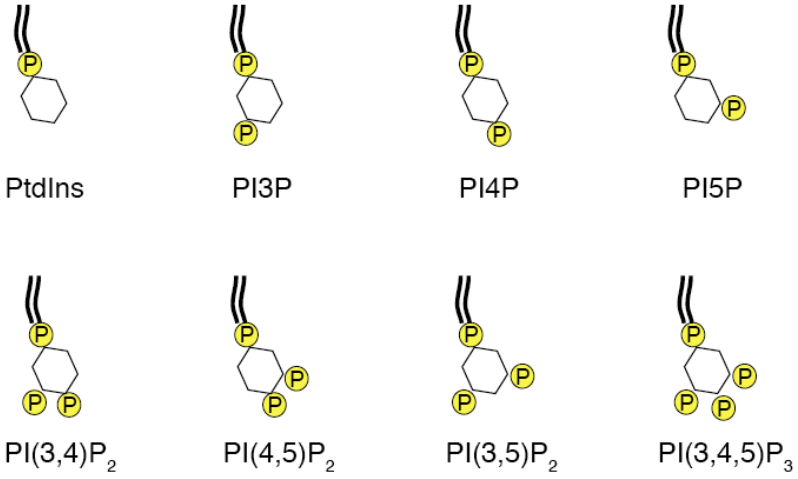

PI-Kinase
Effector
P
HO
OH
PtdIns
PIP
Effectors
FYVE
PX
PH
ENTH
PI-Phosphatase

Figure 1.1 Phosphatidylinositol and the phosphoinositide cycle

(A) Chemical structure of phosphatidylinositol (PtdIns).

(B) Phosphoinositide (PIP) is generated by phosphorylation of PtdIns by PI kinases. PIP is converted back to PtdIns by PI phosphatases. PIP recruits and activiates downstream effector proteins through a set of PIP binding motifs, such as FYVE, PX, PH, ENTH domains.

Figure 1.2


PtdIns
PI3P
PI4P
PI5P
PI(3,4)P2
PI(4,5)P2
PI(3,5)P2
PI(3,4,5)P3

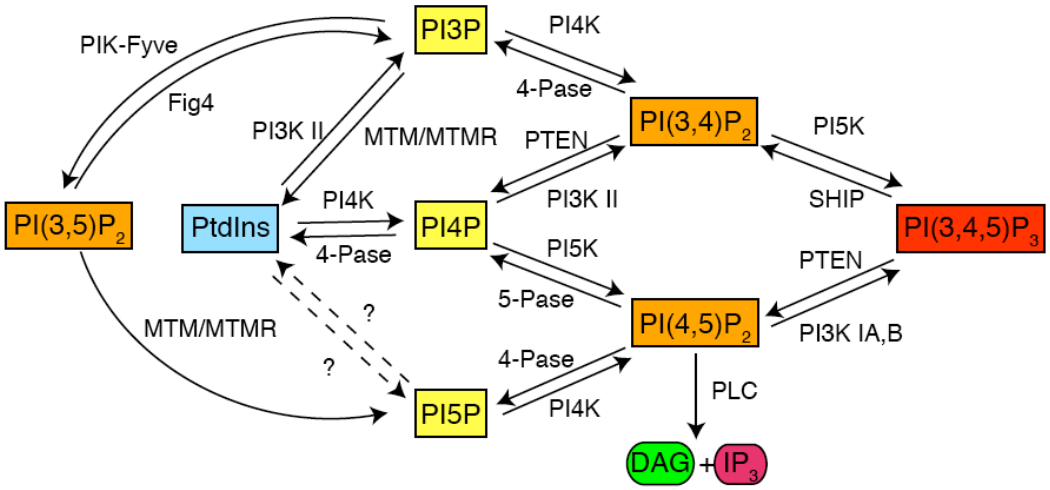

PIK-Fyve
Fig4
PI3K II
MTM/MTMR
PI3P
PI4K
4-Pase
PI(3,4)P2
PI5K
PTEN
PI3K II
SHIP
PI(3,5)P2
PtdIns
PI4K
4-Pase
PI4P
PI(3,4,5)P3
PI5K
5-Pase
PTEN
PI3K IA,B
MTM/MTMR
?
?
PI(4,5)P2
4-Pase
PI4K
PI5P
PLC
DAG
+
IP3

Figure 1.2 The metabolic cycle of phosphoinositides

PtdIns and PIPs are inter-converted by a set of PI kinases and PI phosphatases in cells. $PI(4,5)P_2$ can be metabolized by other enzymes, such as phospholipase C (PLC), to generate the second messengers diacylglycerol (DAG) and inositol-trisphosphate (IP_3).

Figure 1.3

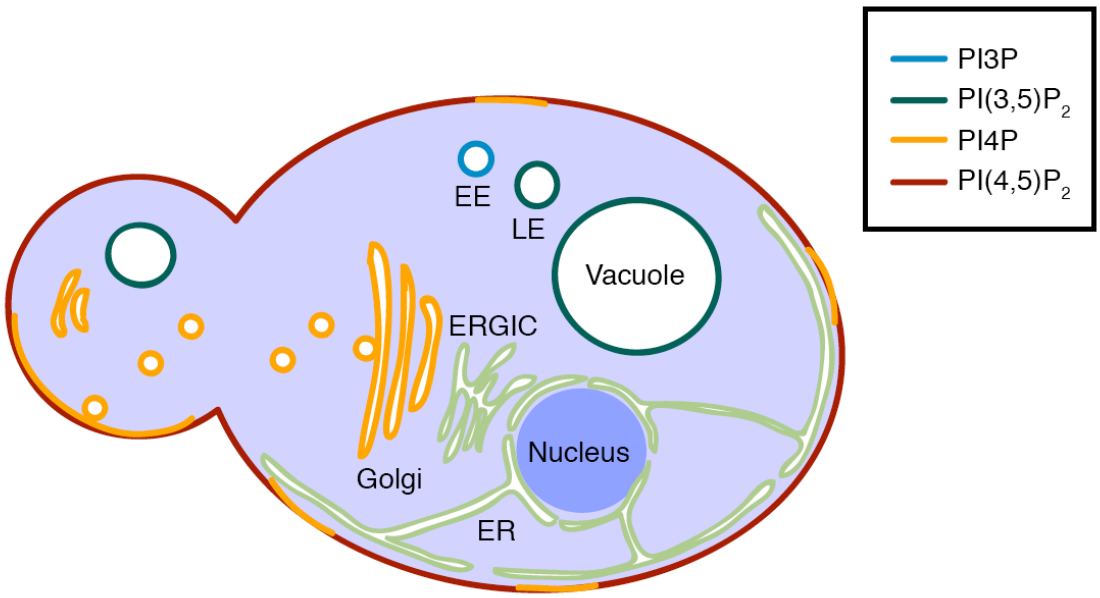

PI3P
PI(3,5)P2
PI4P
PI(4,5)P2
EE
LE
Vacuole
ERGIC
Nucleus
Golgi
ER

Figure 1.3 Compartment specific localization of PIPs in the budding yeast *Saccharomyces cerevisiae*

PI3P is enriched at early endosomes (EE), $PI(3,5)P_2$ is enriched at late endosomes (LE) and the vacuole, PI4P is localized at the Golgi and the plasma membrane, $PI(4,5)P_2$ is predominantly localized at the plasma membrane. ER, endoplasmic reticulum. ERGIC, endoplasmic reticulum-Golgi intermediate compartment.

A variety of important cellular processes are regulated by PIPs, including membrane trafficking (Vicinanza et al, 2008), cytoskeletal remodeling (Martin, 1998), ion transport (Suh & Hille, 2008), and control of vacuole size and homeostasis (Cooke, 2002). Consistent with their unique localizations, studies have revealed that different PIPs have distinct functions in cells. For example, PI3P directly controls endosomal and vacuolar membrane trafficking (Katzmann et al, 2003; Simonsen et al, 1998). PI4P regulates the organization and dynamics of the Golgi network, formation of secretory vesicles, as well as the functions of certain Golgi-localized proteins (Audhya et al, 2000; Natarajan et al, 2009). $PI(3,5)P_2$ is involved in the regulation of vacuole morphology and size in yeast and plant cells in response to certain stress conditions (Efe et al, 2005; Gary et al, 1998; Yamamoto et al, 1995). In mammalian cells, $PI(3,5)P_2$ metabolism has been linked to neuronal degenerative disorders (Chow et al, 2007; Zhang et al, 2007). Of the seven PIP species, $PI(4,5)P_2$ has attracted a broad interest as it directly controls a plethora of important cellular processes at the plasma membrane. In this thesis, I focus on the regulation of $PI(4,5)P_2$ signaling.

The cellular functions of $PI(4,5)P_2$

$PI(4,5)P_2$ as a second messenger precursor

$PI(4,5)P_2$ represents a focal point of PIP signaling as it is the precursor of several important second messengers. $PI(4,5)P_2$ is cleaved by phospholipase C (PLC) upon stimulation at the plasma membrane to generate diacylglycerol

(DAG) and inositol trisphosphate (IP_3) (Figure 1.2). IP_3 is a small, water-soluble molecule that leaves the plasma membrane and diffuses rapidly through the cytoplasm. It can bind and open the IP_3-mediated calcium channels in the ER membrane, thus leading to the release of calcium ions stored in the ER lumen into the cytoplasm to activate many downstream signaling pathways. The other cleavage product, DAG, remains associated with the plasma membrane, where it can recruit and activate protein kinase C (PKC) and lead to the phosphorylation of downstream target proteins.

$PI(4,5)P_2$ can be further phosphorylated at position D3 on the inositol ring to generate $PI(3,4,5)P_3$ in higher eukaryotic cells. $PI(3,4,5)P_3$ directly binds and activates the protein kinase Akt, which activates downstream anabolic signaling pathways required for cell growth and survival. $PI(3,4,5)P_3$ has also been shown to mediate a variety of cellular processes, including cell proliferation, migration, chemotaxis and cell differentiation (Henle et al, 2011; Srinivasan et al, 2003; Wieman et al, 2007). High levels of $PI(3,4,5)P_3$ have been linked to severe diseases including diabetes and cancer (Marone et al, 2008). Clinical studies have revealed that a number of genes controlling the metabolism of $PI(3,4,5)P_3$ are either oncogenes or tumor suppressors; therefore, it is of practical significance to understand how $PI(3,4,5)P_3$ and $PI(4,5)P_2$ levels are balanced in the cell. Importantly, $PI(4,5)P_2$ itself directly recruits and regulates several effector proteins at the PM to control cell growth and polarity, cell signaling, and membrane trafficking pathways.

PI(4,5)P_2 regulates actin cytoskeleton

PI(4,5)P_2 interacts with and modulates the function of numerous actin regulatory proteins to control the assembly of actin cytoskeleton at several levels (Yin & Janmey, 2003). First, PI(4,5)P_2 regulates the initiation of actin filament assembly by stimulating the WASP family proteins, which activate the Arp2/3 complex, mediating actin nucleation and filament branching (Campellone & Welch, 2010). Second, PI(4,5)P_2 facilitates the disassociation of capping proteins from the plus end of actin filaments to stimulate actin polymerization. The capping protein CapZ and actin monomer binding protein, cofilin, are also inhibited by PI(4,5)P_2, which facilitates actin polymerization (dos Remedios et al, 2003). Studies have also shown that PI(4,5)P_2 inhibits gelsolin, a potent actin filament severing protein that weakens the non-covalent bonds between actin molecules to break the filament, by dissociating gelsolin from actin *in vitro* (Sun et al, 1999). Third, PI(4,5)P_2 activates a number of proteins, including vinculin, talin and ERM proteins, to bind and link actin to trans-membrane receptors at the plasma membrane (Sechi & Wehland, 2000). The binding to PI(4,5)P_2 by these proteins is essential to transmit PM signals for efficient actin cytoskeletal remodeling at localized sites.

PI(4,5)P_2 regulates membrane trafficking

PI(4,5)P_2 has been shown to be directly involved in membrane trafficking processes along the secretory and endocytic pathways. PI(4,5)P_2 is an important regulator for both clathrin-dependent and clathrin-independent endocytosis

(Donaldson, 2003; Jost et al, 1998). Studies have shown that $PI(4,5)P_2$ induces a conformational change in the adaptor protein 2 complex (AP-2), that is required for its interaction with sorting signals of trans-membrane proteins (Bonifacino & Traub, 2003; Gaidarov & Keen, 1999; Honing et al, 2005). Loss of synaptojanin isoforms, the $PI(4,5)P_2$ 5–phosphatases, resulted in the accumulation of clathrin-coated pits and vesicles, suggesting that the membrane concentration of $PI(4,5)P_2$ controls the stability of endocytic clathrin coats (Harris et al, 2000). Moreover, $PI(4,5)P_2$ interacts with many other proteins functioning in the endocytic pathway, including AP180, epsin and dynamin, to control the clustering, recognition and invagination of the cargo proteins at the plasma membrane (Bethoney et al, 2009; Ford et al, 2002; Mao et al, 2001; Schmid et al, 1998). In addition, the dynamic assembly and disassembly of actin cytoskeleton is directly involved in multiple stages of endocytosis. As discussed above, a number of proteins related to the function of the actin cytoskeleton, such as WASP and profilin, are regulated by $PI(4,5)P_2$.

In addition to endocytosis, $PI(4,5)P_2$ also directly regulates exocytosis. In 1995, Hay and colleagues reported that $PI(4,5)P_2$ is involved in the ATP-dependent and Ca^{2+}-triggered exocytosis of dense-core vesicles (DCV). They found that the addition of $PI(4,5)P_2$-specific antibodies or PLC strongly inhibited regulated DCV exocytosis in permeabilized PC12 cells (Hay et al, 1995). In addition, $PI(4,5)P_2$ has been shown to regulate SNARE-dependent membrane fusion (James et al, 2008), as James and colleagues found that the Ca^{2+}-dependent activator protein for secretion (CAPS) stimulates SNARE-dependent

fusion in a $PI(4,5)P_2$-dependent manner. Furthermore, a recent study showed the exocyst complex is regulated by $PI(4,5)P_2$. In yeast cells, the exocyst is a protein complex involved in the tethering and spatial targeting of post-Golgi vesicles to the plasma membrane prior to vesicle fusion. Liu and colleagues found the exocyst subunit Exo70 associates with the PM through its direct interaction with $PI(4,5)P_2$, which in turn regulates the tethering and fusion of exocytosed vesicles with the plasma membrane (Liu et al, 2007). The authors also identified key conserved residues at the C-terminus of Exo70 that are crucial for the interaction of Exo70 with $PI(4,5)P_2$. Disrupting the Exo70-$PI(4,5)P_2$ interaction abolished the membrane association of Exo70 and inhibited the docking and fusion of post-Golgi secretory vesicles. However, it did not block their transport to the PM. Together, these findings have shown that $PI(4,5)P_2$ is a key regulator of multiple steps in endocytosis and late stages in exocytosis.

$PI(4,5)P_2$ and human diseases

In addition to actin cytoskeletal organization and membrane trafficking, many other cellular roles for $PI(4,5)P_2$ have been described. For instance, $PI(4,5)P_2$ is also an important regulatory factor in ion channel activation at the plasma membrane (Suh & Hille, 2008), septin assembly during cell division (Bertin et al, 2010; Logan & Mandato, 2006), and transcription in the nucleus (Mellman et al, 2008). All of these discoveries have demonstrated that $PI(4,5)P_2$ regulates numerous aspects of cellular physiology. Therefore it's not surprising that mis-regulation of $PI(4,5)P_2$ metabolism leads to serious diseases.

Mutations in the gene that encodes that PIP 5-phosphatase OCRL, which is recruited to endocytic clathrin coated pits to turn over $PI(4,5)P_2$ during a late stage in endocytosis (Erdmann et al, 2007), is the cause of Lowe's syndrome, a multisystem disorder characterized by anomalies affecting the eye, the nervous system and the kidney. A recent study indicated that $PI(4,5)P_2$ levels are mis-regulated in cells from Alzheimer's disease (AD) patients (Berman et al, 2008). It is believed that accumulation of the Aβ peptide mediates many aspects of AD pathogenesis. Berman and colleagues found that incubation of primary cortical neurons with oligomeric Aβ decreases cellular $PI(4,5)P_2$ levels. In addition, they noticed that the inhibitory effect of Aβ on hippocampal long-term potentiation was strongly suppressed when normal $PI(4,5)P_2$ levels were restored in the brain, by down-regulating the activity of the PIP 5-phosphatase synaptojanin 2 (Berman et al, 2008). Recently, many other diseases have been linked to the mis-regulation of $PI(4,5)P_2$ metabolism in cells, including Down's syndrome (Voronov et al, 2008), the lethal contractual syndrome type 3, and psychiatric diseases (McCrea & De Camilli, 2009). In the case of Down's syndrome, it is believed that over-expression of the synaptojanin-1 gene is linked to this disorder. Accordingly, knocking down of synaptojanin-1 suppressed the defects in a mouse model system for Down's syndrome (Voronov et al, 2008).

Synthesis of $PI(4,5)P_2$ by PIP kinases

Because numerous cellular processes are regulated by $PI(4,5)P_2$, the generation and turnover of $PI(4,5)P_2$ must be precisely regulated. The biosynthesis of $PI(4,5)P_2$ is catalyzed by PIP 5-kinases, while the turnover of $PI(4,5)P_2$ is regulated by PIP 5-phosphatases and phospholipases (Figure 1.2). While all of these enzymes are equally important for $PI(4,5)P_2$ homeostasis in the cell, in this thesis I will particularly focus on the regulation of PIP 5-kinases, especially the yeast PI4P 5-kinase Mss4.

PIP kinase activity was initially discovered by Hokin and Hokin in the early 1960s (Hokin & Hokin, 1964). The PIP kinases were later purified and characterized from erythrocytes (Bazenet et al, 1990; Ling et al, 1989). The initial purification separated PIP kinase activity into two pools, designated as type I and type II respectively. In red blood cells, the type I PIP kinase is a peripheral membrane protein, whereas the type II PIP kinase is largely cytoplasmic (Bazenet et al, 1990). Later it was found that although type I and II PIP kinases both synthesize $PI(4,5)P_2$, they utilize different substrates; type I PIP kinase uses PI4P as the precursor to synthesize $PI(4,5)P_2$ while the type II PIP kinase utilizes PI5P (Rameh et al, 1997). Considering the extremely low level of PI5P in the cell, the majority of $PI(4,5)P_2$ is generated by the type I PIP 5-kinases. While all PIP kinase subfamilies demonstrate some substrate flexibility *in vitro*, they exhibit a relatively high specific activity toward one PIP species *in vivo*. This indicates that the synthesis of distinct phosphoinositide isoforms is highly regulated through the

selective localization of a phosphoinositide kinase at specific membranes inside the cell that are enriched in its substrate as well as other trans-regulating factors.

To better understand how PIP kinases function, Rao and colleagues solved the structure of type II β PIP kinase (Rao et al, 1998). Based on the primary amino acid sequence, there is significant structural homology between PIPKIIβ and protein kinases. This structural homology lies within the ATP binding site of the PIP kinase and additional conserved residues within the PIP kinase spatially align with the catalytic residues of protein kinases, such as protein kinase A (PKA). Three catalytic residues are absolutely identical among the PKA and the PIP kinases; K150, D278, and D369 in PIPKIIβ (e.g. K72, D166 and D184 in PKA); K150 corresponds to the lysine that binds the α-phosphate of ATP in the protein kinases, D278 functions as a weak base in kinase catalysis and D369 binds catalytic Mg^{2+} or Mn^{2+} similar to its counterpart in protein kinases (Rao et al, 1998). As expected, substitutions of these residues result in the complete loss of PIP kinase activity.

Protein kinases have highly variable sequences that determine their specificity toward protein substrates; these sequences are therefore called specificity loops. In PIP kinases, there are also specificity loops (also referred to as activation loops). These specificity loops are conserved within each subfamily, but otherwise divergent between PIP 3-, 4-, and 5-kinase isoforms (Kunz et al, 2000). Kunz and colleagues found that when the specificity loops of PIPKIβ (a

PI4P 5-kinase) and PIPKIIβ (a PI5P 4-kinase) were swapped, each chimera changed its substrate specificity. This illustrates that a specificity loop of a PIP kinase defines its specificity toward different PIP species (Kunz et al, 2000). Interestingly, the authors also found that the specificity loop contributes to the sub-cellular localization of PIP kinases, as the PIPKIIβ-Iβloop mutant (the specificity loop in PIPKIIβ was replaced by the specificity loop of PIPKIβ in the mutant) would target to the plasma membrane where PIPKIβ normally locates. In contrast, the PIPKIβ-IIβloop mutant became cytoplasmic similar to the cytoplasmic localization for PIPKIIβ. These results clearly demonstrate that the specificity loops of PIP kinases are also important for their sub-cellular distribution. However, whether the kinase-substrate is essential for the localization of PIP kinases was not explored. As part of my thesis research, I have examined whether the localization of a type I PIP 5-kinase is controlled by changes in levels of its substrate, PI4P. Protein-protein interactions also seem to be involved in the selective localization of PIP kinases. PIPKI isoforms (α, β and γ) have unique sub-cellular distribution although their specificity loops are highly homologous. Future studies are needed for a more complete understanding of what proteins are required and/or sufficient for the spatial targeting of these PIP kinases. As a second focus for my thesis research, I have employed both genetic and biochemical approaches to identify and characterize novel regulators of PIP kinase activity and targeting.

Regulation of PIPKIs in mammalian cells

The activities of PIPKIs are tightly regulated to synthesize localized pools of $PI(4,5)P_2$ necessary to execute specific functions. A number of PIPKI interacting proteins have been identified in mammalian cells and have been shown to regulate PIPKI kinase activity, including AP-2 at sites of endocytosis (Krauss et al, 2006), talin at tight junctions (Di Paolo et al, 2002), and some small GTPases such as ARF6 (Honda et al, 1999). A number of other factors, such as phosphatidic acid (PA) and post-translational modifications are also reported to regulate PIPKI activities, as summarized below.

Regulation of PIPKIs by AP-2 and talin

$PI(4,5)P_2$ stimulates a conformational change within AP-2 that is required for its interaction with sorting signals of trans-membrane proteins and its stable association with the plasma membrane (Bonifacino & Traub, 2003; Gaidarov & Keen, 1999; Honing et al, 2005). Interestingly, Krauss and colleagues discovered that the μ2 subunit of AP-2 directly interacts with the kinase domain of PIPKIs *in vitro* and in native protein extracts (Krauss et al, 2006). In addition to this finding, they observed that endocytic cargo binding to the μ2 subunit leads to a potent stimulation of PIPKI activity. Thus a positive feed-forward loop consisting of endocytic cargo proteins, the AP-2 μ2 subunit and PIPKIs may provide a specific pool of $PI(4,5)P_2$ at the plasma membrane that regulates AP-2 dependent cargo internalization.

Another PM protein that interacts and regulates PIPKI is talin. Talin is an important component of focal adhesions that links integrin to the actin cytoskeleton (Critchley, 2000). Interestingly, Di Paolo and colleagues identified talin as an interacting partner of the PIPKIγ90 splice variant (Di Paolo et al, 2002). PIPKIγ90 was targeted to focal adhesions in non-neuronal and brain cells by interacting through its carboxyl terminus with the FERM domain of talin. Moreover, the authors observed that this interaction significantly stimulates the PIP kinase activity of PIPKIγ90 in an *in vitro* PIP kinase assay. Over-expression of PIPKIγ90, or expression of its C-terminal domain, disrupts the assembly of focal adhesions, suggesting that maintaining local $PI(4,5)P_2$ levels at focal adhesions is important for their stability.

Regulation of PIPKIs by small GTPases

Small GTPases regulate a variety of signaling events in cells. Accordingly, PIPKIs were found to be downstream effectors of Rho GTPases in mammalian cells (Shibasaki et al, 1997). RhoA, Rac1 and Cdc42, three members of Rho GTPase family, can stimulate all three PIPKI isoforms (α, β and γ) to synthesize $PI(4,5)P_2$ both *in vitro* and *in vivo* (Tolias et al, 2000; Weernink et al, 2004). Interestingly, RhoA and Rac1, but not Cdc42, interact with all three PIPKI isoforms independent of their GTP binding status. One study suggested the activation of PIPKI by RhoA might be mediated through the RhoA effector, Rho-kinase (ROCK) (Oude Weernink et al, 2000). Binding of PIPKI isoforms to the GTP-bound, but not GDP-bound, RhoA could be displaced by ROCK, suggesting

that direct and constitutive PIP5K-RhoA binding does not trigger PIPKI activation (Weernink et al, 2004). Rac1 has been shown to regulate actin cytoskeletal remodeling through PIPKI (Tolias et al, 2000). The interaction between Rac1 and PIPKI requires the C-terminal polybasic region of Rac1(Tolias et al, 2000). Intriguingly, a point mutation in the C-terminus of Rac1 that disrupts PIPKI binding also negatively affects Rac1-induced actin polymerization in permeabilized platelets (Tolias et al, 2000). Consistent with this observation, the direct interaction between PIPKI and Rac1 resulted in a modest increase in the PIPKI activity (Tolias et al, 2000). The polybasic region of Rac1 also interacts with $PI(4,5)P_2$ and this interaction is important for the PM localization of Rac1. Therefore, Rac1, PIPKI and $PI(4,5)P_2$ might all contribute to the Rac1-induced actin polymerization in a positive feed-forward loop.

ADP ribosylation factors (ARFs) are a family of small GTPases that control vesicle biogenesis and trafficking in cells (Aikawa & Martin, 2005). Honda and colleagues found that over-expression of ARF6 leads to an increase of $PI(4,5)P_2$ levels at the PM, and ARF6 can interact with and activate PIPKI in the presence of phosphatidic acid (Honda et al, 1999). Over-expression of a constitutively active form of ARF6 (Q67L) induces the formation of large internal vesicles rich in $PI(4,5)P_2$ and coated with actin (Aikawa & Martin, 2003; Aikawa & Martin, 2005), which is also observed when PIPKI is over-expressed in cells. The formation of $PI(4,5)P_2$-containing vesicles induced by PIPKI over-expression is

not blocked by the expression of a dominant-negative form of ARF6. This suggests that PIPKIs are activated and function downstream of ARF6.

Regulation of PIPKIs by PA and phospholipase D

Phosphatidic acid (PA) was first found to stimulate the activity of PIPKI *in vitro* (Jenkins et al, 1994). Consistent with this, a previous study demonstrated that PA enhances the binding affinity of PIPKIβ for its substrate PI4P (Jarquin-Pardo et al, 2007). PA is generated through the hydrolysis of phosphatidylcholine (PtdCho) by phospholipase D (PLD). Interestingly, $PI(4,5)P_2$ is a cofactor for PLD activation. In addition, PLD is also activated by the small GTPases, such as ARF6 (Powner & Wakelam, 2002). A model has been proposed in which ARF6 activates both PLD and PIPKI to generate PA and $PI(4,5)P_2$. PA generated by PLD activates PIPKI and $PI(4,5)P_2$ generated by PIPKI in turn activates PLD (Oude Weernink et al, 2007). This positive feed-forward loop leads to the increased synthesis of PA and $PI(4,5)P_2$. The localized increase of $PI(4,5)P_2$ and PA may then initiate endocytosis in specific regions of the plasma membrane (Brown et al, 2001).

Regulation of PIPKIs by phosphorylation

Phosphorylation of serine, threonine and tyrosine residues is a common regulatory mechanism to control protein enzymatic activity. Accordingly, phosphorylation of PIPKIs may affect the interaction between PIPKI with its binding partners, and/or it may result in a conformational change of PIPKI that

affects its kinase activity. A previous study showed that PIPKIα is phosphorylated by cyclic AMP-dependent protein kinase (PKA), which leads to attenuation of its activity (Park et al, 2001). Serine 214 was found to be a major PKA-phosphorylation site in PIPKIα. Park et al. also found PIPKIα activity was induced upon dephosphorylation by an okadaic acid-sensitive phosphatase, protein phosphatase 1 (PP1). Consistent with this observation, dephosphorylation of PIPKIα with PP1 increased its PIP kinase activity *in vitro* (Park et al, 2001).

Studies have revealed that another PIP kinase, PIPKIγ90, also undergoes phosphorylation *in vivo* and has two adjoining phosphorylation sites in its C-terminus, Y649 and S650 (Lee et al, 2005; Ling et al, 2003). The phosphorylation of residue S650 in PIPKIγ90 impairs its interaction with talin. At synapses in neuronal cells, S650 is phosphorylated by Cdk5 and mitogen-activated protein kinase (MAPK) at rest, and dephosphorylated by calcineurin upon stimulation (Cousin & Robinson, 2001; Gong et al, 2005). S650 is also a substrate for Cdk1, and its phosphorylation in mitosis correlates with focal adhesion disassembly. Phosphorylation of the adjacent Y649 in PIPKIγ90 by Src kinase was suggested to directly increase the affinity of PIPKIγ90 for talin (Ling et al, 2002). However, it is also likely that phosphorylation of Y649 inhibits the phosphorylation at S650, which subsequently drives the disassociation of talin from PIPKIγ90 (Lee et al, 2005). Together, these results showed phosphorylation may affect the interaction between PIPKIs and their binding partners.

All PIPKI isoforms also have intrinsic protein kinase activity and autophosphorylate themselves (Itoh et al, 2000). PIPKI autophosphorylation is markedly enhanced *in vitro* by the addition of PtdIns, which leads to a significant inhibition of PIPKI lipid kinase activity (Itoh et al, 2000). This observation indicates that the lipid kinase activity of PIPKI might be regulated by PtdIns-dependent autophosphorylation *in vivo* as well.

Regulation of Mss4, the PIPKI in budding yeast

A number of PIP kinases detected in higher eukaryotic cells are conserved in the budding yeast *Saccharomyces cerevisiae* (Figure 1.4). *S. cerevisiae* has proven to be a very useful model organism to study PIP metabolism and PIP function. The gene *MSS4* encodes the only PIPKI in budding yeast cells. *MSS4* was initially isolated as a multi-copy suppressor of the temperature-sensitive growth of the *stt4-1*^{ts} mutant. Stt4 is the PM-localized PI 4-kinase in yeast. This suggests Mss4 and Stt4 at least partially function in the same signaling pathway (Yoshida et al, 1994). Following characterization of Mss4 demonstrated that it displays PI4P 5-kinase activity both *in vitro* and *in vivo* (Desrivieres et al, 1998). Expression of the mammalian type I PIP kinase, but not the ype II PIP kinase, could restore the viability of yeast cells harboring a *mss4* null mutation (Homma et al, 1998), further suggesting Mss4 is the PIPKI homolog in yeast. The majority of Mss4 localizes to the plasma membrane as illustrated by fluorescent microscopy and cell fractionation experiments. Interestingly, rather than being

distributed evenly along the PM, Mss4 clusters at the PM to form punctate structures, which are termed Mss4 PIK (**P**hospho**I**nositide **K**inase) patches (Audhya & Emr, 2002). Mss4 PIK patches are distinct from Stt4 PIK patches as implicated by their limited colocalization at the PM (Audhya & Emr, 2002). As part of my thesis research work, I have characterized the regulatory mechanisms that govern Mss4 PIK patch assembly and dynamics.

In addition to its peripheral association with the PM, a pool of Mss4 is also detected in the nucleus upon over-expression (Audhya & Emr, 2003). A temperature sensitive *mss4-1* mutant allele accumulating in the nucleus at restrictive temperature was isolated by Audhya and Emr. This study showed that the nuclear entry of Mss4 is mediated by the karyopherin Kap123, as cells lacking Kap123 no longer accumulated Mss4-1-GFP in the nucleus at the restrictive temperature. No nuclear fluorescent signal was detectable when GFP-Mss4 was over-expressed in *kap123* null mutant cells. The export of Mss4 out of the nucleus is mediated by the *BCP1* gene product, as over-expression of *BCP1* could restore the growth of *mss4-1* mutant allele at the restrictive temperature and target Mss4-1-GFP back to the PM. Based on these results, the authors proposed a model for Mss4 regulation by shuttling between the nucleus and the cytoplasm (Audhya & Emr, 2003). However, a nuclear role of Mss4 remains unknown.

Figure 1.4

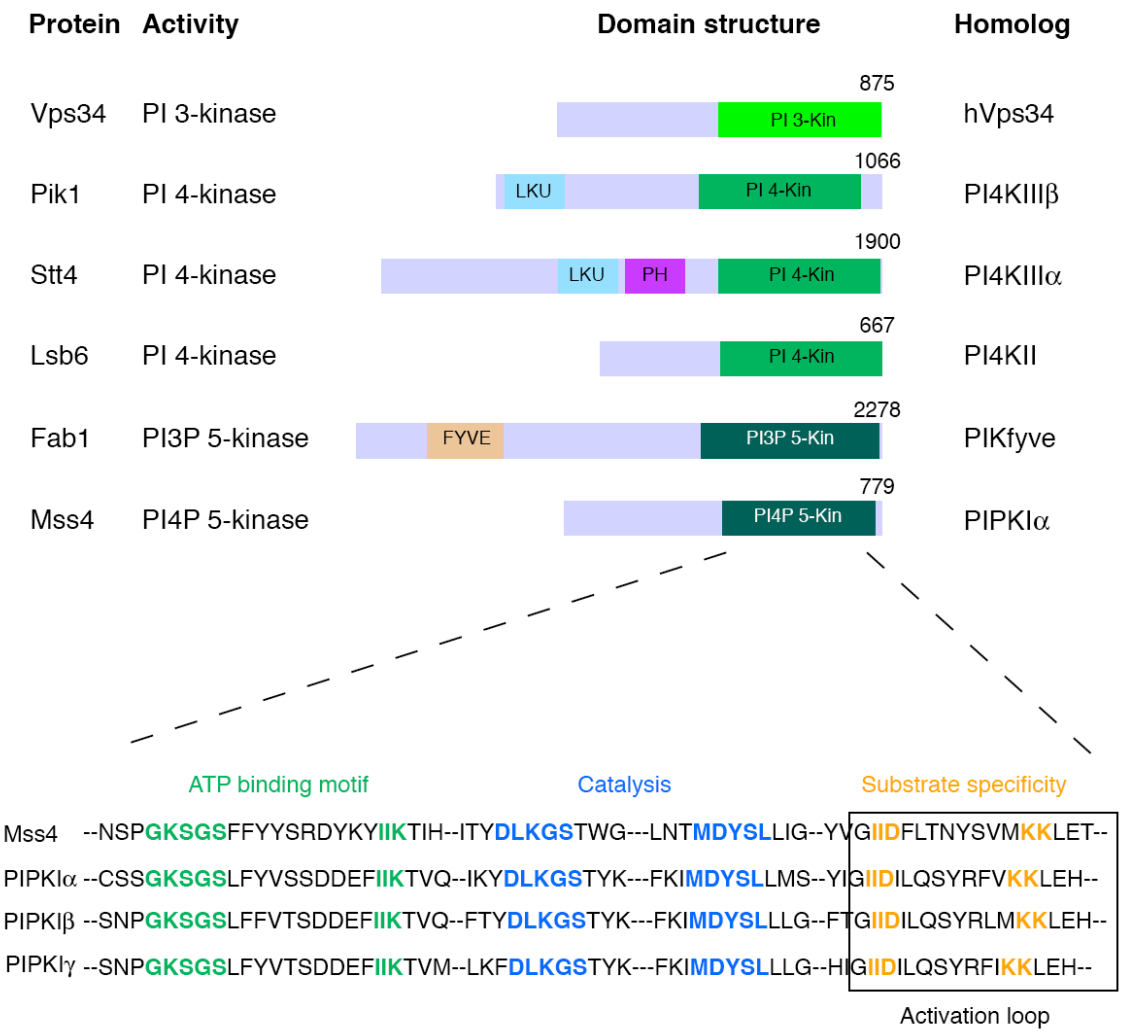

Protein
Activity
Domain structure
Homolog
Vps34
PI 3-kinase
875
PI 3-Kin
hVps34
Pik1
PI 4-kinase
1066
LKU
PI 4-Kin
PI4KIIIβ
Stt4
PI 4-kinase
1900
LKU
PH
PI 4-Kin
PI4KIIIα
Lsb6
PI 4-kinase
667
PI 4-Kin
PI4KII
Fab1
PI3P 5-kinase
2278
FYVE
PI3P 5-Kin
PIKfyve
Mss4
PI4P 5-kinase
779
PI4P 5-Kin
PIPKIα
ATP binding motif
Catalysis
Substrate specificity
Mss4 --NSPGKSGSFFYYSRDYKYIIKTIH--ITYDLKGSTWG---LNTMDYSLLIG--YVGIIDFLTNYSVMKKLET--
PIPKIα --CSSGKSGSLFYVSSDDEFIIKTVQ--IKYDLKGSTYK---FKIMDYSLLMS--YIGIIDILQSYRFVKKLEH--
PIPKIβ --SNPGKSGSLFFVTSDDEFIIKTVQ--FTYDLKGSTYK---FKIMDYSLLLG--FTGIIDILQSYRLMKKLEH--
PIPKIγ --SNPGKSGSLFYVTSDDEFIIKTVM--LKFDLKGSTYK---FKIMDYSLLLG--HIGIIDILQSYRFIKKLEH--
Activation loop

Figure 1.4 PIP kinases in the budding yeast *Saccharomyces cerevisiae*

Vps34 is the PI 3-kinase responsible for PI3P synthesis. Pik1, Stt4 and Lsb6 are the PI 4-kinases responsible for PI4P synthesis. Fab1 is the PI3P 5-kinase synthesizing $PI(3,5)P_2$. Mss4 is the PI4P 5-kinase synthesizing $PI(4,5)P_2$. The residues that make up catalytic core of the PIP kinase family are highlighted with color in the Mss4 lipid kinase domain and conserved in all PIP kinases. PH, pleckstrin homology domain. LKU, lipid kinase unique domain.

At the start of my thesis research work, very little was known about the regulation of Mss4 PIP kinase activity at the PM. Studies from the Hall lab suggested that Mss4 functions downstream of calmodulin, a small Ca^{2+} binding protein that is involved in controlling a variety of physiological processes (Desrivieres et al, 2002). *MSS4* over-expression specifically suppresses the mutant phenotype of a calmodulin allele defective in actin cytoskeleton organization (*cmd1-226*), but not those defective in calmodulin localization or nuclear division. In addition, the *cmd1-226^{ts}* mutant displays defects in endocytosis, that are similar to those seen in *mss4* mutants. Consistent with these observations, $PI(4,5)P_2$ levels were decreased in the *cmd1-226^{ts}* strain at the restrictive temperature (Desrivieres et al, 2002). These data suggested that calmodulin functions upstream of Mss4 in a pathway that controls actin cytoskeleton organization and endocytosis via $PI(4,5)P_2$. Though calmodulin and Mss4 seem to function at least partially in the same signaling pathway, it is not clear whether calmodulin directly interacts with Mss4 or regulates other proteins like calmodulin-dependent kinases/phosphatases to modulate Mss4 PIP kinase activity. A study of the mammalian PI4P 5-kinase, PIPKIγ, suggested that the dephosphorylation of PIP kinases by calceneurin stimulates $PI(4,5)P_2$ synthesis (Nakano-Kobayashi et al, 2007).

Smaczynska-de and colleagues found that Arf3, the yeast homolog of ARF6, modulates $PI(4,5)P_2$ levels at the PM to facilitate endocytosis

(Smaczynska-de et al, 2008). In support of this, studies in the Emr lab showed that loss of the Arf3 GEF Syt1 was synthetically lethal with the *mss4^{ts}* mutation (Audhya et al, 2004). In addition, deletion of the Arf3 GAP Gcs1 rescues the growth defect of *mss4^{ts}* cells at the nonpermissive temperature (Emr lab, unpublished results). These results suggest that Arf3 GTPase functions as a positive regulator of PI(4,5)P_2 synthesis *in vivo*, similar to its homolog ARF6 in mammalian cells. However, Arf3 is not an essential protein required for Mss4 PIK patch assembly at the PM (Ling Y., unpublished results).

Phosphatidylinositol/phosphatidylcholine transfer proteins (PITPs) are thought to regulate PIP synthesis. Sec14, the major yeast PITP, has been shown to facilitate PI4P synthesis by the type IIIβ PI4K Pik1 *in vivo* (Routt et al, 2005; Schaaf et al, 2008). Sec14 is essential in yeast cells, and interestingly, the requirement of Sec14 is bypassed by the loss of the PI4P phosphatase Sac1 (Rivas et al, 1999). Consistent with this observation, loss of Osh4 protein, which stimulates Sac1 phosphatase activity (Stefan et al, 2011), rescues the growth defect of *sec14*Δ cells and *pik1* mutant cells (Fairn et al, 2007; Fang et al, 1996; Li et al, 2002). *Saccharomyces cerevisiae* encodes another five proteins that are highly homologous to Sec14 and therefore are named as Sec Fourteen Homologues 1-5 (*SFH1-5*). A previous study found that over-expression of Sfh4 and Sfh5 resulted in a 10% and 50% increase in PI(4,5)P_2 levels respectively, with no apparent effect on other PIP levels (Routt et al, 2005). It is not known whether these PITPs interact with Mss4 and directly enhance Mss4 PIP kinase

activity *in vivo*. In stead, the current model is that PITPs may present substrate PtdIns for modification by the the PI 4-kinases, leading to increased rates of PI4P synthesis (Mousley et al, 2012). These pools of PI4P may then be used by PI4P 5-kinases, such as Mss4, to generate $PI(4,5)P_2$.

Sphingolipids have also been implicated to function upstream of the Mss4 PIP 5-kinase (Kobayashi et al, 2005). *MSS4* was identified as a multicopy suppressor of myriocin-induced cell death in yeast. Myriocin is an inhibitor of the early sphingolipid synthesis enzymes Lcb1 and Lcb2. Kobayashi and colleagues found myriocin treatment of yeast affects both the activity and the sub-cellular localization of Mss4. The authors further showed that when phytosphingosine (PHS) was added back to myriocin treated-cells, the normal localization of Mss4 was restored. In addition, in *csg2*Δ cells that are deficient in mannosylated inositol-phosphorylcermide (MIPC) synthesis, Mss4 mis-localized from the PM. However, Mss4 still localized to the PM in *ipt1*Δ cells that cannot synthesize $M(IP)_2C$ from MIPC. These data suggest that the loss of the sphingolipid species MIPC was responsible for the mis-localization of Mss4. However, the underlying mechanisms of how sphingolipids target Mss4 to the PM still remain elusive, as sphingolipids are known to be enriched in the outer leaflet of the plasma membrane. Intriguingly, sphingolipids are known to regulate important PM protein cascades in yeast, such as Tor2 signaling. Future studies are needed to address whether Tor signaling regulates Mss4 function.

The above mentioned studies revealed some interesting aspects of Mss4 PIK patch assembly and PIP kinase activity regulation. However, still little is known on how Mss4 assembles into PIK patches at the PM and how its activity is regulated at these structures.

Overview of the thesis

$PI(4,5)P_2$ is an essential signaling lipid that regulates a variety of critical cellular processes. Maintaining proper levels of $PI(4,5)P_2$ at the plasma membrane is crucial for cell growth and development. Mis-regulation of $PI(4,5)P_2$ metabolism leads to many serious human diseases. It is therefore essential to understand how cells regulated $PI(4,5)P_2$ metabolism through PIPKI activity. My thesis research work has focused on two major aims: (1) study how the lipid kinase Mss4 is assembled into PIK patches at the plasma membrane, (2) study how Mss4 PIK kinase activity is controlled at PIK patches. First, I have characterized some general features of Mss4 PIK patches, including their dynamics at the PM and the oligomerization status of Mss4. Second, I discovered that the substrate for Mss4, PI4P, and the kinase domain of Mss4 are necessary for the assembly of Mss4 PIK patches at the PM. Third, I have found that a large N-terminal region of Mss4 functions as a negatively regulatory domain for PIP kinase activity. To identify additional regulators of Mss4, I used a quantitative proteomic approach (SILAC-MS) and identified Opy1 as an Mss4 interacting protein. Opy1 is a tandem PH domain-containing protein that partially colocalizes with Mss4 at PIK patches. Genetic and biochemical experimental

results indicated that Opy1 binds PI(4,5)P_2 and Mss4 at the PM and negatively regulates Mss4 PIP kinase activity at PIK patches. Finally, in the last chapter of my thesis, I have discussed the major findings of my research including some unpublished work that may provide important insight into the regulation of PI(4,5)P_2 signaling in the future.

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Chapter 2

Characterization of Mss4 PIK Patches[1]

Introduction

PIK patches are large protein complexes consisting of multiple copies of lipid kinases along with other regulatory proteins oligomerized into a nonuniform punctate structure on cellular membranes. The biological functions of PIK patches are not completely clear. However, based on our studies we hypothesized that PIK patches are platforms for PIP synthesis, where multiple lipid kinases can be coordinately regulated by accessory factors such as small GTPases, protein kinases/phosphatases to control local PIP levels at these sites. Previous studies revealed that both the PtdIns 4-kinase Stt4 and the PI4P 5-kinase Mss4 form PIK patches at the plasma membrane. However, unlike Stt4 PIK patches, which are relatively static structures with a lifetime over 3 minutes, Mss4 PIK patches are very dynamic at the plasma membrane. In addition, Mss4 PIK patches do not co-localize with Stt4 PIK patches, suggesting the mechanisms for the regulation of the PtdIns 4-kinase and PI4P 5-kinase complexes are distinct. Although it has been shown Mss4 functions in many signaling pathways (mostly via its product $PI(4,5)P_2$) *in vivo*, how Mss4 PIK patches are assembled at the PM and how Mss4 PIP kinase activity is regulated by other factors are still largely unexplored questions. In this chapter, I will focus

[1] Some of the experiments presented in this chapter were published in Ling Y. *et al*, *EMBO J*, 2012. The dissertation author was the primary investigator and author of the paper.

on the characterization of the general features of Mss4 PIK patches and study how Mss4 PIK patches are assembled at the plasma membrane. In addition, I will show Mss4 PIP kinase activity is controlled by phosphorylation events at PIK patches.

Materials and Methods

Yeast strains and Plasmid Construction

A list of all *Saccharomyces cerevisiae* strains and plasmids used in this study and their genotypes can be found in Supplementary Tables S1 and S2. Homologous recombination was used to tag or delete genes in yeast (Longtine et al, 1998) . All integrations were verified by PCR analysis and expression of fusion proteins was confirmed by Western Blot analysis. Gene subcloning was performed by PCR using Ex Taq (Takara) or KOD Polymerase (Novagen) and subsequent ligation into the designated expression vector with T4 DNA ligase (Fermentas). The yeast shuttle vectors used in this study have been previously described (Sikorski & Hieter, 1989). Restriction enzymes were purchased from New England Biolabs (Ipswich, MA).

Fluorescence Microscopy and Quantification of Fluorescence Intensity

Yeast cells expressing fluorescent fusion proteins were grown to mid-log in synthetic media. Microscopy was performed using a fluorescence microscope (DeltaVision RT; Applied Precision) equipped with FITC and rhodamine filters. Images were captured with a digital camera (Cool Snap HQ; Photometrics) and

deconvolved using softWoRx 3.5.0 software (Applied Precision). For quantification of numbers of Mss4-GFP molecules in PIK patches, cells co-expressing Mss4-GFP and Cse4-3xGFP were grown to mid log phase in YNB media. Cells were examined by fluorescence microscopy and the original, undeconvolved data was used for quantification of intensities. Regions of interest (with identical areas) that contained a single Mss4 PIK patch or Cse4-3xGFP structure were selected and fluorescence intensities were quantified using the Data Inspector tool in the softWoRx 3.5.0 software (Applied Precision). Background signal was determined at the mid section of cells and subtracted from measured fluorescence intensities of PIK patches and Cse4-3xGFP. For comparing PIK patch fluorescence intensity, cells expressing Mss4-GFP or N-terminally truncated Mss4$^{\Delta 2\text{-}346}$ -GFP were grown to mid log phase in YNB media. Cells expressing Mss4-GFP were labeled with FM4-64 (a marker for vacuole membranes) and cells expressing N-terminally truncated Mss4$^{\Delta 2\text{-}346}$ -GFP were labeled with Hoechst dye (a nuclear marker) to distinguish between cell types. Cells were then mixed together and examined by fluorescence microscopy in the same field.

In Vivo Analysis of Phosphoinositides

Phosphoinositides levels were analyzed as previously described (Baird et al, 2008; Stefan et al, 2002). Briefly, 5 OD_{600} units of cells (per strain) were labeled with 50μCi of myo-[2-^{3}H]inositol (Perkin Elmer) in synthetic media lacking inositol for 1 hour. After precipitation in 4.5% perchloric acid (final concentration) for 5

minutes, phospholipids were deacylated by incubation in methylamine reagent (10.7% methylamine, 45.7% methanol, 11.4% 1-butanol) for 50 minutes at 53^{o}C. Excess methylamine was removed by drying in a vacuum chamber, followed by two washes (resuspension by sonication and subsequent drying) and a final resuspension of the pellet in 300 μL sterile water. Subsequently, an equal volume of extraction reagent (1-butanol/ethyl-ether/formic acid ethyl ester at a ratio of 20:4:1) was added and [^{3}H]glycero-phosphoinositides were extracted into the aqueous phase by vortexing for 5 minutes and centrifugation at 14,000 x g for 3 minutes. The extraction was repeated twice more and the final aqueous phase was collected and dried as above. For quantitative analyses, dried pellets were resuspended in sterile water and 5 x 10^{6} cpm of sample was separated on a Partisphere SAX column (Whatman, Florham Park, NJ) attached to a Shimadzu HPLC system (Shimadzu Manufacturing, Kyoto, Japan) and a 610TR on-line radiomatic detector (Perkin Elmer, Waltham, MA) using Ultima Flo scintillation fluid (Perkin Elmer). The HPLC and on-line detector were controlled with EZStart 7.2.1 and ProFSA 3.3 software, respectively, with final data analysis taking place in the latter.

Analysis of Cellular Protein Expression Levels

5 OD_{600} equivalents of mid-log cells pretreated at the indicated temperatures were harvested by precipitation in 10% trichloroacidic acid (TCA). Precipitates were washed in acetone, aspirated, resuspended in lysis buffer (150 mM NaCl, 50mM Tris pH 7.5, 1mM EDTA, 1% SDS), and mechanically lysed with glass

beads. Protein sample buffer (150 mM Tris pH 6.8, 6M Urea, 6%SDS, 10% β-mercaptoethanol, 20% Glycerol) was added and extracts were analyzed by SDS-PAGE and immunoblotting with anti-G6PDH (Sigma) and anti-GFP (Santa Cruz Biotechnology) antibodies.

In Vivo Crosslinking and Coimmunoprecipitation

Yeast cells expressing epitope-tagged proteins were grown in synthetic media to an OD_{600} of 0.5-0.8. Cells were harvested and spheroplasted with Zymolyase 100T (Seikagaku Biobusiness). After spheroplasting, cells were washed and then crosslinked by 1mM Dithiobis [succinimidyl propionate] (DSP, Pierce) in crosslinking buffer (20mM HEPES, 0.7M Sorbitol, 100mM KOAc pH=7.4) for 30 mins. 100mM Tris-HCl (pH=7.5) was added to the crosslinking buffer to stop the reaction. Cells were lysed by vortexing with glass beads in 200uL Urea cracking buffer (6M Urea, 50mM Tris-HCl, 1mM EDTA, 1% SDS, pH=7.5). Cell lysates were diluted in 5mL IP buffer (50mM Tris-HCl, 150mM NaCl, 5mM EDTA, 0.5% Tween-20, pH=7.5) with protease inhibitors added and cleared by centrifugation at 13,000xg for 10 mins twice. IPs were done by adding either HA or Flag affinity gel (Sigma-Aldrich) to the supernatant and incubated for 1-2h at 4°C. The affinity matrix was washed by the IP buffer four times. Finally, CoIPs were eluted and reduced with elution buffer (100mM Tris-HCl pH=8.0, 1% SDS, 10mM DTT) at 65°C. Elutes were mixed with equal volume of sample buffer (6M Urea, 150mM Tris-HCl pH=6.8, 6% SDS, Bromophenol Blue) and analyzed by SDS-PAGE and immunoblotting.

Recombinant Protein Expression and Purification

The bacterial expression vectors pGEX6P-1 (GE healthcare) and ppSUMO (Sondermann lab) were used to generate recombinant fusion proteins. C41(DE3) cells (Lucigen) transformed with protein expression plasmids were grown at 37°C to an OD_{600} of 0.4. The bacteria were then shifted to 25°C for 1 h and protein expression was induced by the addition of 0.1 mM IPTG for 16 hours. GST and His_6 fusion proteins were purified from C41 cells with glutathione sepharose 4B (GE Healthcare) and Ni-NTA agarose (Qiagen) respectively according to the manufactures instructions. Protein concentrations were determined by the Bradford assay (BioRad). Purified proteins were stored at -80^{o}C in phosphate buffered saline (PBS) or in storage buffer (PBS, 50% glycerol) until further use.

Lipid Binding Assays

The lipid overlay assays were performed essentially as described (Stefan et al, 2011). Briefly, 150 pmol of lipids solubilized in chloroform were spotted onto reinforced nitrocellulose (Whatman) as indicated. Membranes were blocked with 5% non-fat milk in PBS containing 0.1 % Tween 20 and incubated overnight with 5 μg/ml His_6-SUMO-$Mss4^{454-779}$ constructs in PBS containing 0.1 % Tween 20 and 2% fatty acid-free albumin. Membranes were extensively washed with PBS containing 0.1 % Tween 20 and bound protein was detected by immunoblotting using anti-His_6-Tag (Novagen) against the His_6 tag in the His_6-SUMO-$Mss4^{454-779}$ fusion protein.

Results

Mss4 forms dynamic protein complexes, PIK patches, at the PM

Mss4 is a cytoplasmic protein that associates with the inner face of the PM (Audhya & Emr, 2003). To understand how Mss4 is regulated, we employed cells solely expressing a functional Mss4-GFP fusion. Mss4-GFP formed cortical, punctate structures at the PM: PIK patches (Figure 2.1A, observed at both mid and top sections of cells) similar to previously reported patterns for Mss4 localization (Audhya & Emr, 2002; Smaczynska-de et al, 2008; Sun et al, 2007). Reconstructions (2D projections of Z series) of cells expressing Mss4-GFP indicated that there are approximately ~30-50 Mss4 PIK patches in each individual yeast cell. However, the oligomeric status or the dynamics of Mss4 PIK patches at the PM has not been addressed. To determine whether multiple copies of Mss4 assemble at PIK patches, we tested if an Mss4-13x*myc* fusion isolated with an Mss4-3xHA fusion in co-immunoprecipitation experiments. Mss4-13x*myc* was present in anti-HA immuno-precipitates from cell lysates co-expressing Mss4-3xHA, but not from control cell lysates lacking Mss4-3xHA (Figure 2.1B), suggesting that Mss4 oligomerizes. To estimate the copy numbers of Mss4 molecules present in PIK patches, we expressed Mss4-GFP in cells co-expressing a Cse4-3xGFP fusion (both are integrated; Figure 2.1C). Cse4-3xGFP forms a complex containing 96 GFP molecules in the nucleus of yeast cells (Markus et al, 2009). By comparing the GFP signal intensities of Mss4 PIK patches and Cse4-3xGFP in the nucleus, we found a distribution of ~5 to 30

copies of Mss4-GFP in each PIK patch (Figure 2.1C). However, most PIK patches contained ~10-20 Mss4-GFP molecules (Figure 2.1C). Thus, multiple copies of the Mss4 lipid kinase assemble at PIK patches.

To address the dynamics of Mss4 PIK patches, we performed time-lapse imaging experiments following Mss4-GFP at the cell surface by focusing on the top of cells. Strikingly, Mss4 PIK patches were highly dynamic and short-lived structures. More than 75% of Mss4 PIK patches appear to change localization within 30 seconds (Figure 2.1D), and the lifetime of Mss4 PIK patches range from 10 to 40 seconds (Figure 2.1E). This dramatic rearrangement in distribution likely occurs by the dynamic assembly and disassembly of Mss4 PIK patches as well as lateral movements along the surface of the PM, as Mss4 PIK patches did not move into the interior of the cell by following Mss4-GFP in mid-sections of cells (Data not shown).

Figure 2.1

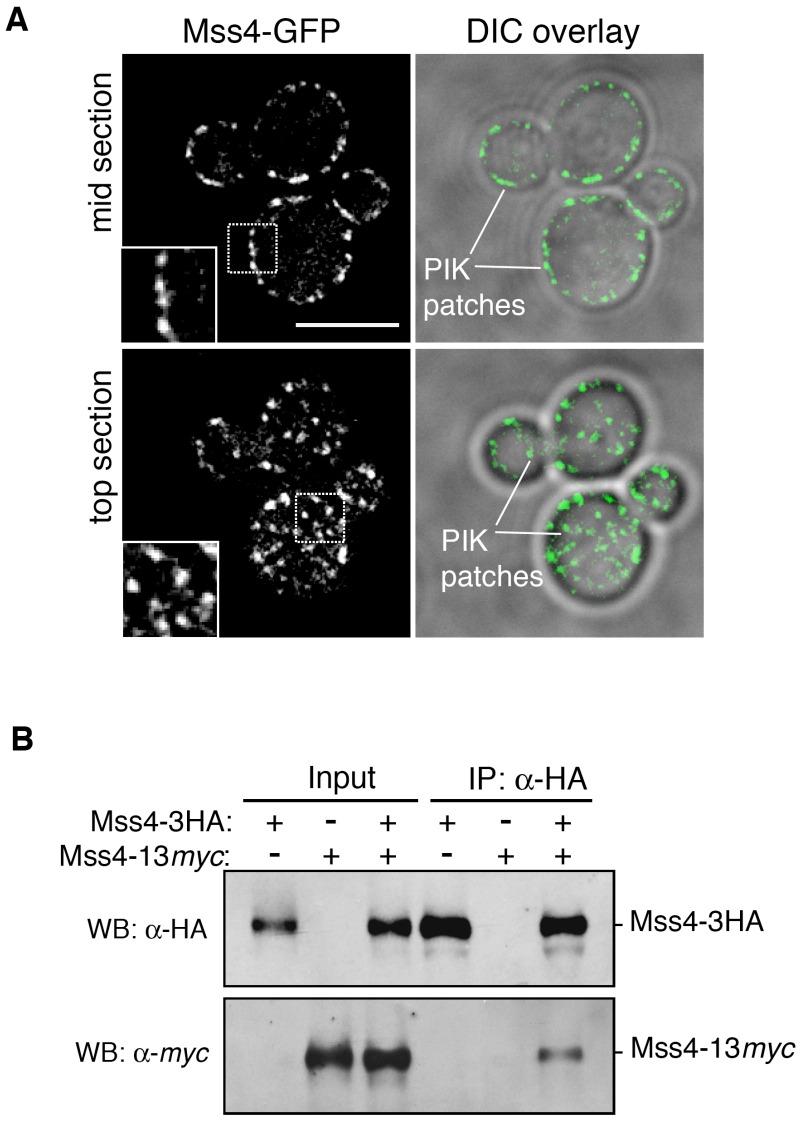
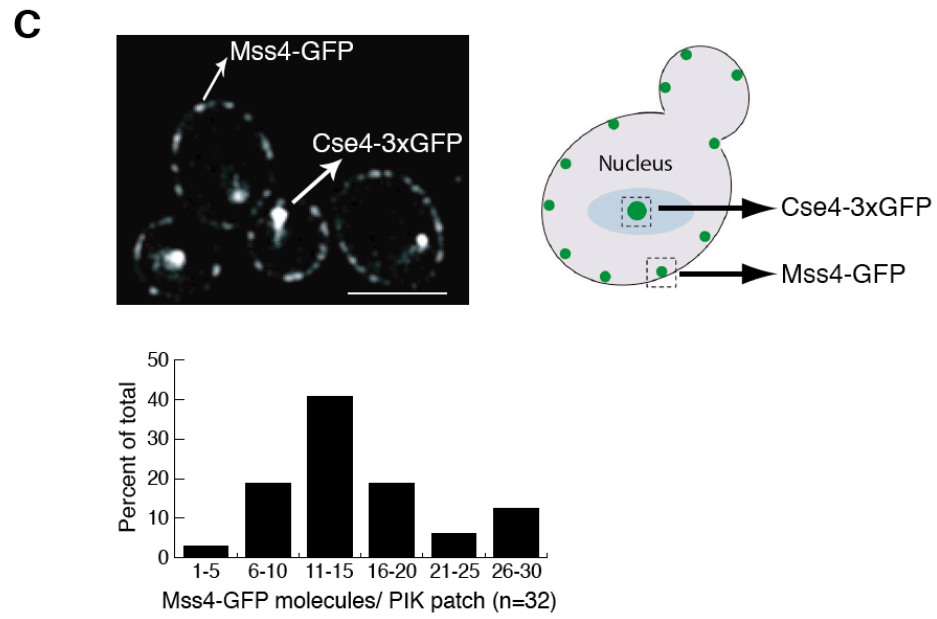
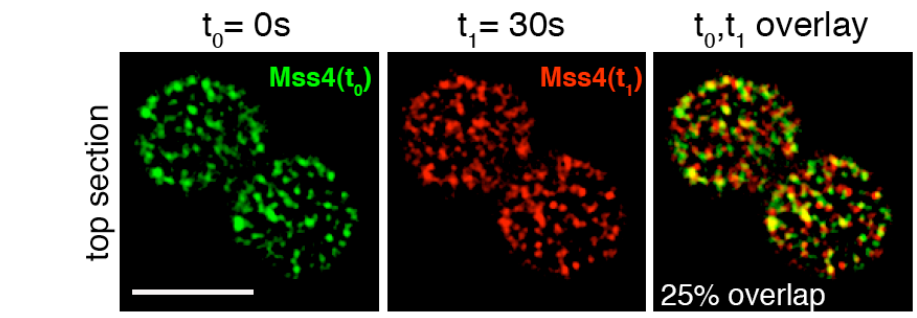

A
Mss4-GFP
DIC overlay
mid section
top section
PIK
patches
PIK
patches
B
Input
IP: α-HA
Mss4-3HA: + - + + - +
Mss4-13myc: - + + - + +
WB: α-HA
Mss4-3HA
WB: α-myc
Mss4-13myc

Figure 2.1


C
Mss4-GFP
Cse4-3xGFP
Nucleus
Cse4-3xGFP
Mss4-GFP
Percent of total
50
40
30
20
10
0
1-5
6-10
11-15
16-20
21-25
26-30
Mss4-GFP molecules/ PIK patch (n=32)


D
t0= 0s
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top section
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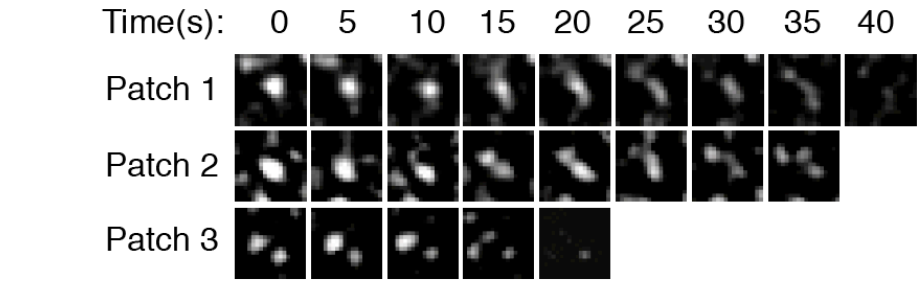
25% overlap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5
10
15
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35
40
Patch 1
Patch 2
Patch 3

Figure 2.1 Mss4 forms oligomeric, dynamic cortical structures at the plasma membrane

(A) Mss4-GFP localization in mid and top sections of yeast cells. Cells shown are representative of over 100 cells observed. Lines indicate individual Mss4 PIK patches at the PM. The inset (boxed area) shows a region magnified two-fold. Levels of the color images overlayed on DIC images were adjusted with Adobe Photoshop. Scale bar, 5 μm.

(B) Mss4-13myc was co-immunoprecipitated with Mss4-3HA. Lysates from cells expressing Mss4-3HA, Mss4-13myc or both were incubated with crosslinker and immunoprecipitated with anti-HA beads, and analyzed by immunoblotting to detect Mss4-Mss4 interaction.

(C) Quantification of numbers of Mss4 molecules in PIK patches (n=32). Cells co-expressing integrated Mss4-GFP and Cse4-3xGFP were analyzed by fluorescence microscopy. Arrows indicate Mss4-GFP at PM and Cse4-3xGFP in the nucleus respectively. Scale bar, 5 μm. Numbers of Mss4 were calculated based on fluorescence signal intensity of Mss4 PIK patches and Cse4-3xGFP in the nucleus as an internal standard.

(D) Mss4 PIK patches are dynamic structures. Cells expressing Mss4-GFP were examined by time-lapse fluorescence microscopy. Images of Mss4-GFP at the cell surface were captured every 5 seconds. At time t_0=0 sec, Mss4-GFP is shown in green, at t_1=30 sec Mss4-GFP is shown in red. Scale bar, 5 μm.

(E) Three representative examples of Mss4 PIK patch lifetimes at the cell surface, images were captured every 5 seconds.

Mss4 PIK patches are distinct protein complexes at the PM

Several other PM proteins also form punctate structures, including actin patches and eisosomes (Pruyne & Bretscher, 2000a; Pruyne & Bretscher, 2000b; Walther et al, 2006). Eisosomes ('eis' meaning into or portal and 'soma', meaning body) are large, heterodimeric, immobile protein complexes at the plasma membrane which mark the site of endocytosis. Mss4 PIK patches were distinct from cortical actin patches as they did not significantly colocalize with each other; actin patches were highly enriched the daughter cells while Mss4 PIK patches distributed evenly in the mother and daughter cells. In addition, Mss4 PIK patch formation did not require actin polymerization, as Mss4 PIK patches stayed intact when the cells were treated with Latrunculin A, a drug that inhibits actin polymerization efficiently (Figure 2.2A). Likewise, Mss4 PIK patches were distinct from eisosomes as they did not colocalize well revealed by the microscopy experiments (Figure 2.2B). Moreover, deletion of the eisosome core component Pil1, which leads to the collapse of eisosomes, did not affect the assembly of Mss4 PIK patches (Figure 2.2C). These results suggested that Mss4 assembles into unique dynamic structures at the PM. We thus sought to further understand how Mss4 organization and function are regulated.

Figure 2.2

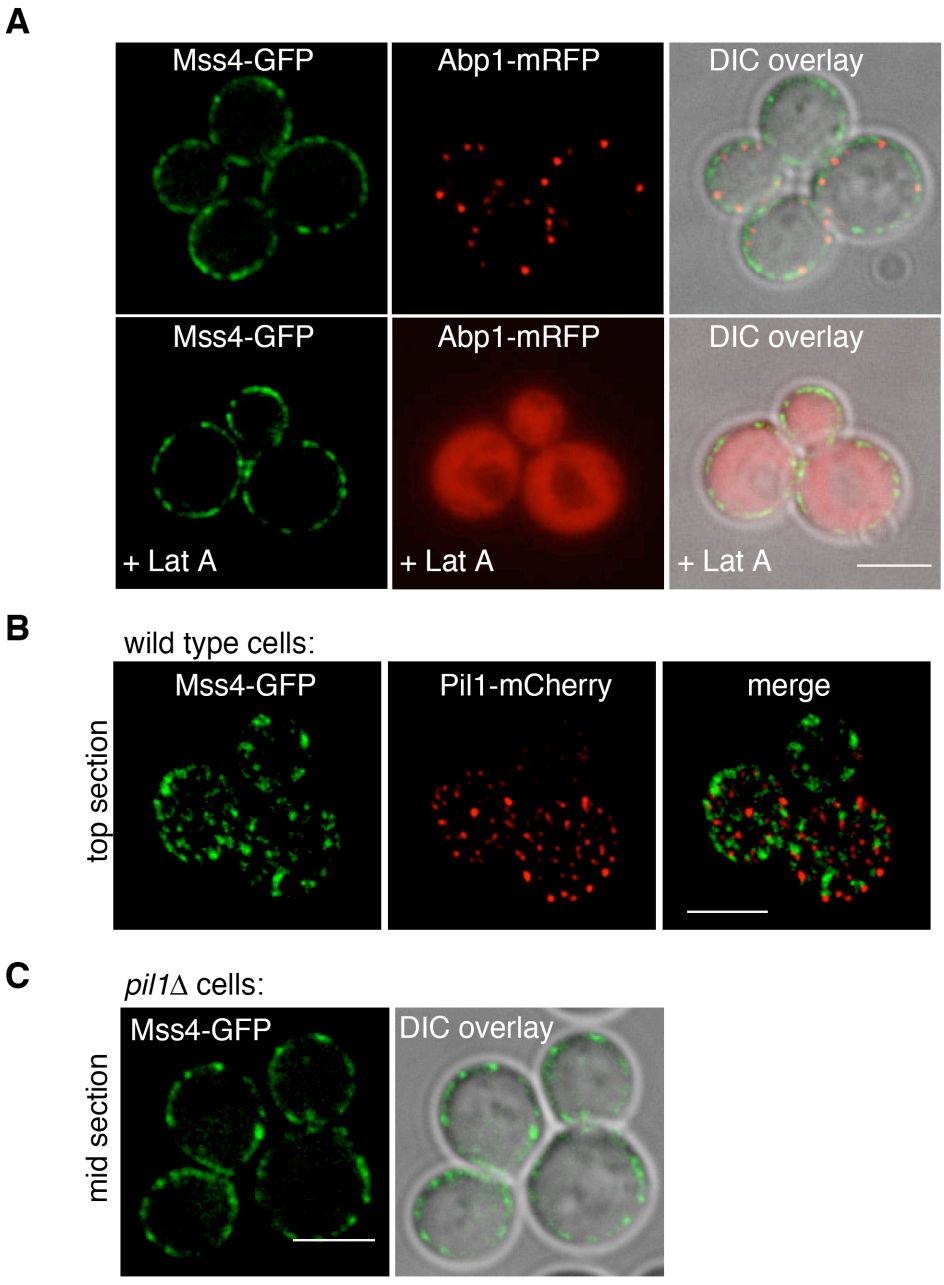

A
Mss4-GFP
Abp1-mRFP
DIC overlay
Mss4-GFP
Abp1-mRFP
DIC overlay
+ Lat A
+ Lat A
+ Lat A
B
wild type cells:
top section
Mss4-GFP
Pil1-mCherry
merge
C
pil1Δ cells:
mid section
Mss4-GFP
DIC overlay

Figure 2.2 Mss4 forms unique, oligomeric structures at the plasma membrane

(A) Localization of Mss4-GFP and Abp1-mRFP in cells. Cells expressing Mss4-GFP and Abp1-mRFP were grown at 26 °C and treated with or without latrunculin A. Scale bar, 5 μm.

(B) Localization of Mss4-GFP and Pil1-mcherry in cells. Scale bar, 5 μm.

(C) Mss4-GFP localization in *pil1*Δ cells. Scale bar, 5 μm.

The Mss4 kinase domain is required for PIK patch assembly

First, I wanted to map the regions/domains of Mss4 that are necessary for Mss4 PIK patch assembly. Mss4 consists of an uncharacterized N-terminal domain, a central nuclear localization signal (NLS), and a conserved C-terminal PIP 5-kinase domain (Figure 2.3A). To map regions in Mss4 necessary for PIK patch assembly, I generated a series of truncated Mss4 mutants tagged with GFP. The large N-terminal region of Mss4 (residues 2-346) and the NLS in Mss4 (residues 347-364) were dispensable for Mss4 PM localization (Figure 2.3A). In contrast, a mutant form lacking the last 54 amino acids of Mss4 (residues 726-779), did not localize to the PM and instead accumulated in the cytoplasm and nucleus (Figure 2.3A). Both Mss4$^{\Delta 726\text{-}779}$-GFP and Mss4$^{\Delta 2\text{-}346}$-GFP were expressed at levels similar to full-length Mss4-GFP (Figure 2.3C).

I then tested if the truncated Mss4-GFP fusions were functional using a plasmid shuffle growth assay. For this, we transformed an *mss4Δ* strain carrying an *URA3*-marked wild type *MSS4* plasmid with plasmids encoding full-length or truncated forms of Mss4-GFP. As expected, cells expressing Mss4$^{\Delta 726\text{-}779}$-GFP alone failed to grow on 5-FOA media due to loss of the *URA3*-marked wild type *MSS4* plasmid. However, neither the N-terminal region nor the NLS were required for Mss4 function, as cells expressing mutant forms of Mss4 lacking these regions were able to grow on 5-FOA plates (Figure 2.3B).

Figure 2.3

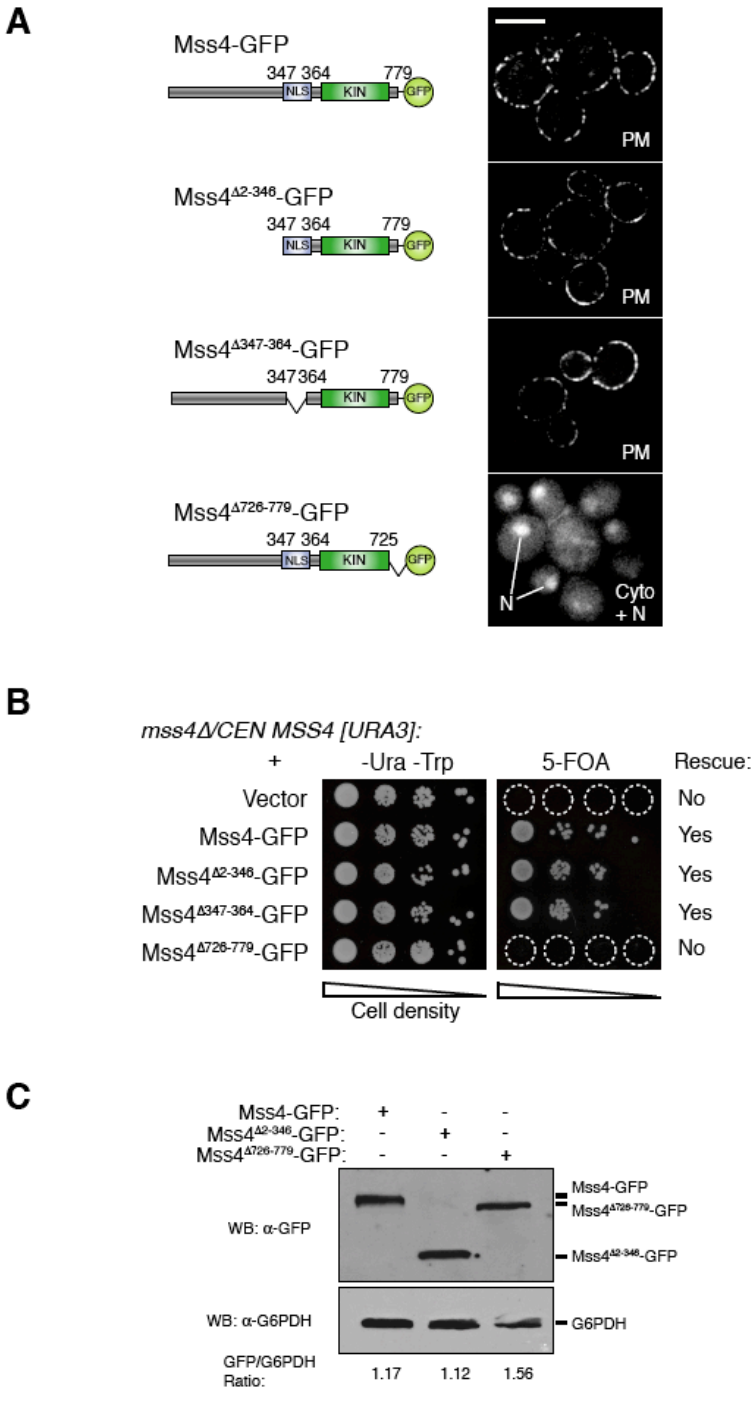

A
Mss4-GFP
347 364
779
NLS
KIN
GFP
PM
Mss4Δ2-346-GFP
347 364
779
PM
Mss4Δ347-364-GFP
347364
779
PM
Mss4Δ726-779-GFP
347 364
725
N
Cyto
+ N
B
mss4Δ/CEN MSS4 [URA3]:
+
-Ura -Trp
5-FOA
Rescue:
Vector
No
Mss4-GFP
Yes
Mss4Δ2-346-GFP
Yes
Mss4Δ347-364-GFP
Yes
Mss4Δ726-779-GFP
No
Cell density
C
Mss4-GFP: + - -
Mss4Δ2-346-GFP: - + -
Mss4Δ726-779-GFP: - - +
WB: α-GFP
Mss4-GFP
Mss4Δ726-779-GFP
Mss4Δ2-346-GFP
WB: α-G6PDH
G6PDH
GFP/G6PDH Ratio:
1.17
1.12
1.56

Figure 2.3 The Mss4 kinase domain is required for PIK patch assembly

(A) Localization of wild type and truncated Mss4-GFP proteins. From top to bottom: full-length Mss4-GFP, N-terminally truncated Mss4-GFP (lacking residues 2-346), NLS-deleted Mss4-GFP (lacking residues 347-364), and C-terminally truncated Mss4-GFP (lacking residues 726-779). Lines indicate the nucleus of cells. Scale bar, 5 μm. Cyto, cytoplasm, N, nucleus; NLS, nuclear localization signal; PM, plasma membrane.

(B) Complementation assays of the truncated Mss4 mutants. The *mss4Δ cells* carrying a centromeric *URA3*-marked *MSS4* plasmid was co-transformed with plasmids expressing various mutant Mss4-GFP forms as indicated. Cells were spotted onto –Ura -Trp plates to retain both plasmids or plates containing 5-FOA to select for loss of the *URA3*-marked *MSS4* plasmid; only cells harboring functional *MSS4-GFP* plasmids grew on 5-FOA plates.

(C) Steady-state expression levels of Mss4-GFP, Mss4$^{\Delta 726\text{-}779}$-GFP, and Mss4$^{\Delta 2\text{-}346}$-GFP. G6PDH steady-state expression was monitored as a loading control. The expression ratios of Mss4-GFP, Mss4$^{\Delta 726\text{-}779}$-GFP, and Mss4$^{\Delta 2\text{-}346}$-GFP (adjusted to G6PDH expression) are shown.

PI4P is required for Mss4 PIK patch assembly

The truncation resulting in Mss4 mis-localization occurs in a conserved region of the C-terminus, termed the activation loop (Kunz et al, 2000). The activation loops of mammalian PI4P 5-kinases control substrate recognition and subcellular targeting (Kunz et al, 2000). Substitution of two highly conserved lysine residues to negatively charged aspartate residues in the activation loop resulted in Mss4 mis-localization (Figure 2.4B). In contrast, substitutions with arginine, retaining the positive charge, did not affect Mss4 PM localization (Figure 2.4B), which is consistent with studies of the mammalian PIP5KI.

Though it's reasonable to assume the PI4P plays an important role in determining PIPKI localization, previous studies did not address whether the *in vivo* localization of PIPKI would change upon depletion of PI4P in cells. Stt4 and Pik1 are the two major PI 4-kinases responsible for PI4P synthesis in yeast (Audhya et al, 2000). To deplete PI4P, we employed a temperature conditional *stt4*^{ts} *pik1*^{ts} double mutant strain to address this question. Strikingly, Mss4-GFP completely localized to the cytoplasm in *stt4*^{ts} *pik1*^{ts} double mutant cells at the restrictive temperature (Figure 2.4C). However, Mss4-GFP normally localized to the PM in the *stt4*^{ts} and *pik1*^{ts} single mutant cells at the non-permissive temperature (data not shown), suggesting that both PI 4-kinases contribute to Mss4 PM targeting. To confirm the direct interaction between Mss4 and PI4P, we purified the Mss4 kinase domain and carried out the protein-lipid overlay assay. And as expected, Mss4 kinase domain binds strongly to PI4P (and PI3P *in vitro*),

but weakly to PI(3,5)P_2 and PI(4,5)P_2 and does not bind to PtdIns at all. To rule out the possibility that the mis-localization of Mss4 in *stt4^{ts} pik1^{ts}* double mutant cells at the restrictive temperature was a indirect effect of depleting of PI(4,5)P_2, we examined the localization of a kinase inactive form of Mss4-GFP in *mss4^{ts}* cells. The kinase-dead Mss4 still localized to the PM when PI(4,5)P_2 is depleted in *mss4^{ts}* cells upon shifting to the nonpermissive temperature (Figure 2.4E), suggesting the mis-localization of Mss4 in *stt4^{ts} pik1^{ts}* double mutant cells was indeed due to low PI4P levels at the PM. Thus, we propose that the C-terminus targets Mss4 to the PM by interacting with PI4P.

The N-terminal region of Mss4 functions as a negative regulatory domain

Our initial results implicated the conserved C-terminus of Mss4 in PIK patch assembly. However, the role of the Mss4 N-terminal region remained unclear. We further examined Mss4 regulation by performing ^{3}H-inositol labeling experiments to measure cellular PI(4,5)P_2 levels in *mss4^{ts}* cells co-expressing various forms of Mss4-GFP. As expected, the C-terminal truncated Mss4$^{\Delta 726-779}$-GFP was defective in PI(4,5)P_2 synthesis (Figure 2.5A and Table 1). However, deletion of the N-terminus increased Mss4 activity, as PI(4,5)P_2 levels were ~2-fold higher in *mss4^{ts}* cells expressing Mss4$^{\Delta 2-346}$-GFP compared to cells expressing full-length Mss4-GFP (Figure 2.5A and Table 1). Elevated PI(4,5)P_2 levels are toxic in cells with impaired PI(4,5)P_2 phosphatase activity (Stefan et al, 2002). Expression of the N-terminal truncated form but not full-length Mss4

Figure 2.4

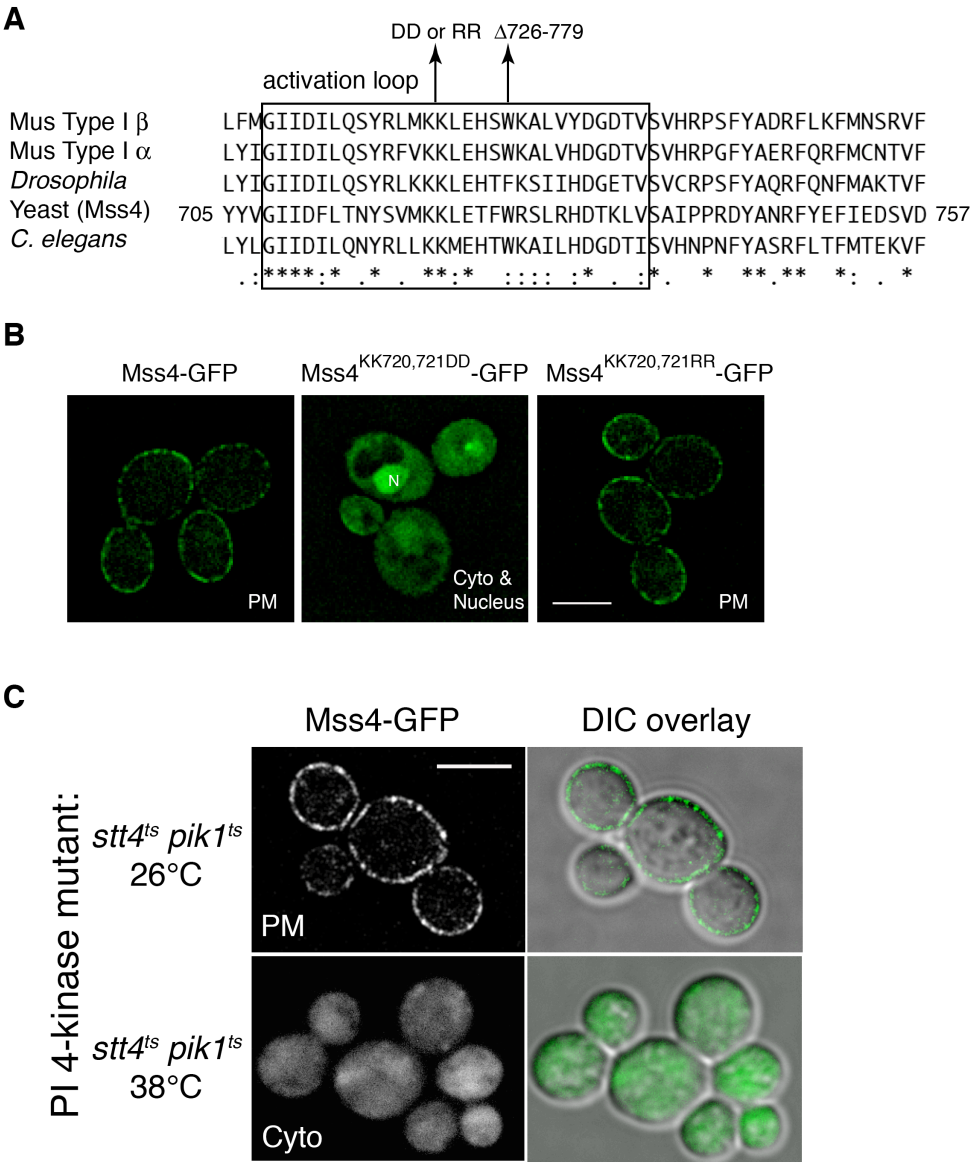

A
DD or RR Δ726-779
activation loop
Mus Type I β
Mus Type I α
Drosophila
Yeast (Mss4)
C. elegans
705
757
B
Mss4-GFP
Mss4KK720,721DD-GFP
Mss4KK720,721RR-GFP
PM
N
Cyto &
Nucleus
PM
C
Mss4-GFP
DIC overlay
PI 4-kinase mutant:
stt4ts pik1ts
26°C
PM
stt4ts pik1ts
38°C
Cyto

Figure 2.4

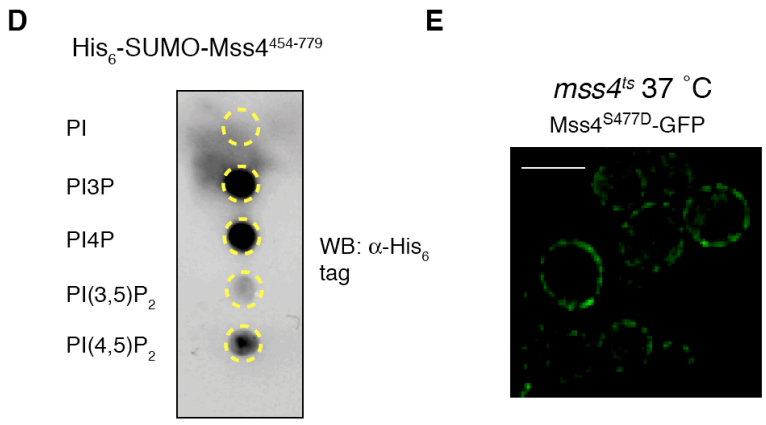


Figure 2.4 PI4P is required for Mss4 PIK patch assembly

(A) Alignment of Mss4 C-terminus with type I PIP kinases from other eukaryotes. The substrate activation loop is highlighted in the boxed area.

* Identical residues; : Conserved residues; . Similar residues.

(B) Localization of wild type Mss4-GFP and Mss4-GFP activation loop mutants Mss4^{KK720,721DD}-GFP and Mss4^{KK720,721RR}-GFP. Cyto, cytoplasm, N, nucleus; PM, plasma membrane. Scale bar, 5 μm.

(C) Mss4-GFP localization in *stt4^{ts} pik1^{ts}* double mutant cells at 26°C and 38°C. Cells were grown at 26 °C to mid log phase and shifted to 38°C for 60 minutes prior to observation by fluorescence microscopy. Scale bar, 5 μm.

(D) The Mss4 kinase domain binds phosphoinositides. PIP lipid overlay binding assays (FAT blots) using His$_6$-SUMO-Mss4$^{454-779}$. Bound protein was detected with antibodies against the His$_6$ tag in the fusion protein.

(E) Mss4^{S477D}-GFP (kinase dead mutant) localization in *mss4^{ts}* cells incubated at 37 °C. Cells were grown at 26 °C to mid log phase, shifted to 37 °C for 60 minutes, and examined by fluorescence microscopy. Scale bar, 5 μm.

Figure 2.5

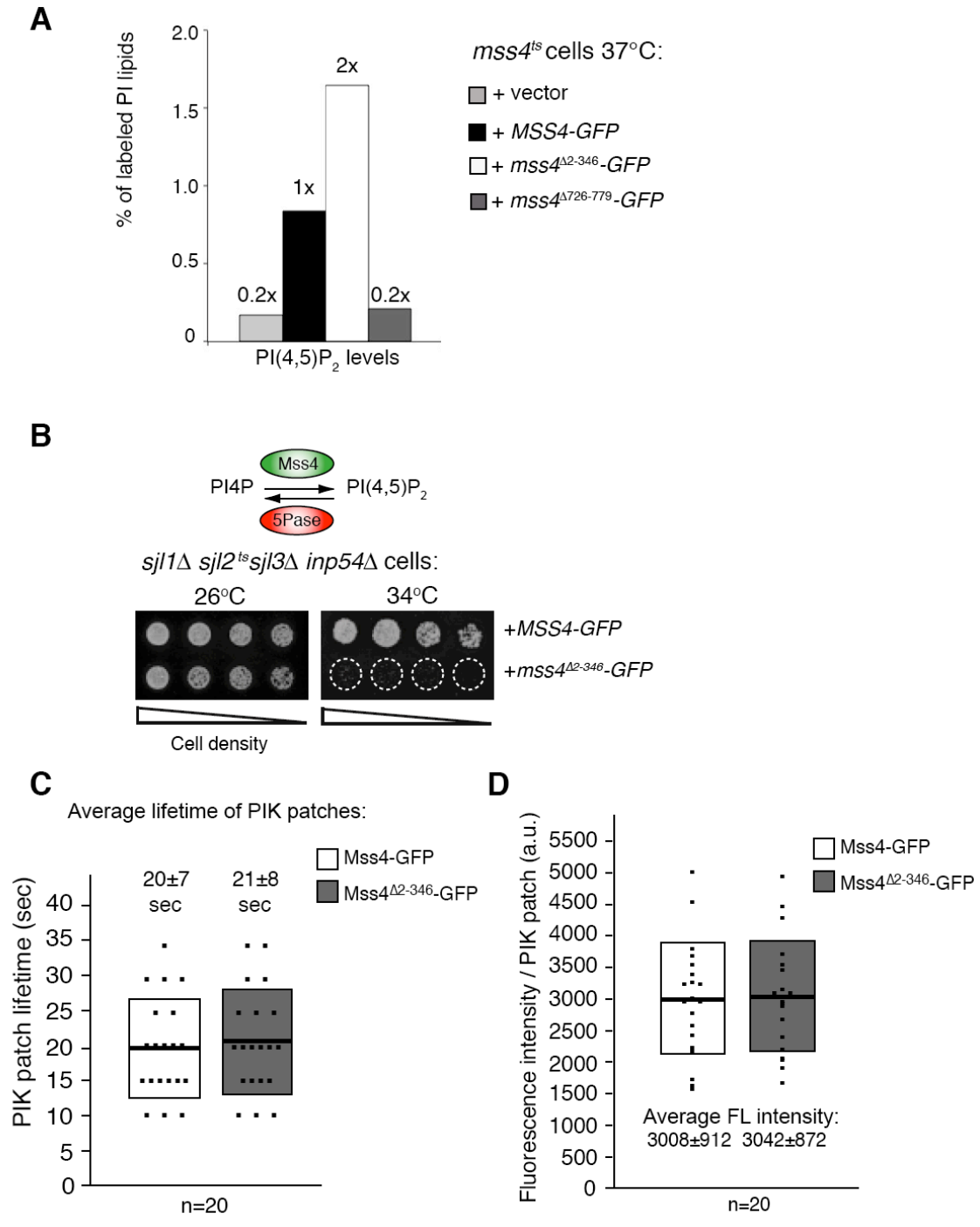

A
% of labeled PI lipids
2.0
1.5
1.0
0.5
0
0.2x
1x
2x
0.2x
PI(4,5)P2 levels
mss4ts cells 37°C:
+ vector
+ MSS4-GFP
+ mss4Δ2-346-GFP
+ mss4Δ726-779-GFP
B
Mss4
PI4P
PI(4,5)P2
5Pase
sjl1Δ sjl2tssjl3Δ inp54Δ cells:
26°C
34°C
+MSS4-GFP
+mss4Δ2-346-GFP
Cell density
C
Average lifetime of PIK patches:
Mss4-GFP
Mss4Δ2-346-GFP
20±7 sec
21±8 sec
PIK patch lifetime (sec)
40
35
30
25
20
15
10
5
0
n=20
D
Mss4-GFP
Mss4Δ2-346-GFP
Fluorescence intensity / PIK patch (a.u.)
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Average FL intensity:
3008±912 3042±872
n=20

Figure 2.5 Mss4 N-terminal region functions as a negative regulatory domain

(A) Cellular PI(4,5)P_2 levels measured by ^{3}H-inositiol labeling and HPLC analysis of *mss4^{ts}* cells expressing empty vector, full-length Mss4-GFP, N-terminally truncated Mss4-GFP (lacking residues 2-346), or C-terminally truncated Mss4-GFP (lacking residues 726-779). The lipid labeling was performed at 37°C, a non-permissive temperature for the *mss4^{ts}* cells. Results from a representative experiment are shown. Additional data are provided in Table 1.

(B) Expression of N-terminally truncated Mss4 impairs the growth of *sjl1*Δ *sjl2^{ts} sjl3*Δ *inp54Δ* cells deficient in PIP 5-phosphatase activity. The *sjl1*Δ *sjl2^{ts} sjl3*Δ *inp54*Δ cells were transformed with full-length or N-terminally truncated *MSS4* plasmids as indicated. Serial dilutions of yeast cells were grown on –Ura plates to retain the *MSS4-GFP* plasmids at either 26°C or 34°C for 4 days.

(C) Measurements of Mss4 PIK patch lifetimes. Mss4-GFP PIK patches in *mss4*Δ cells (white). Mss4$^{\Delta 2\text{-}346}$-GFP PIK patches in *mss4*Δ cells (dark grey). In each experiment, the lines show mean lifetimes measured for twenty PIK patches (n=20) and boxes indicate standard deviation.

(D) Measurements of Mss4 PIK patch fluorescence intensities. Mss4-GFP PIK patches in *mss4*Δ cells (white). Mss4$^{\Delta 2\text{-}346}$-GFP PIK patches in *mss4*Δ cells (dark grey). The lines show mean PIK patch fluorescence intensities measured for twenty PIK patches (n=20) and boxes indicate standard deviation.

Table 1. Phosphoinositide levels in wild type and mutant cells examined				
Strain	**PIP levels (% of total ^{3}H-labeled PtdIns and PIPs)**			
	PI3P	**PI4P**	**PI(3,5)P_2**	**PI(4,5)P_2**
Figure 2.5A				
Exp.1: *mss4^{ts}* 37°C				
+ *Vector*	0.90	0.87	0.08	0.17
+ [*CEN MSS4*]	0.89	0.95	0.06	0.84
+ [*CEN MSS4 $^{\Delta 2\text{-}346}$*]	0.94	0.69	0.05	1.7
+ [*CEN MSS4 $^{\Delta 726\text{-}779}$*]	1.3	1.2	0.18	0.21
Exp. 2: *mss4^{ts}* 37°C				
+ *Vector*	1.2	1.2	0.13	0.22
+ [*CEN MSS4*]	0.63	0.77	0.07	0.65
+ [*CEN MSS4 $^{\Delta 2\text{-}346}$*]	0.86	0.69	0.04	1.7
+ [*CEN MSS4 $^{\Delta 726\text{-}779}$*]	1.1	1.5	0.3	0.23
Figure 2.6B and 2.6C				
mss4^{ts} 37°C				
+ [*CEN MSS4*]	1.1	1.4	0.12	1
+ [*CEN MSS4 ^{S728A}*]	0.87	0.92	0.13	0.14
+ [*CEN MSS4 ^{S728D}*]	0.94	1.16	0.11	0.54
mss4^{ts} 37°C				
+ [*CEN MSS4*]	0.91	1.3	0.23	1.3
+ [*CEN MSS4 ^{S477A}*]	0.83	0.98	0.14	0.45
+ [*CEN MSS4 ^{S477D}*]	0.94	1.05	0.15	0.19
Figure 2.7C				
Exp. 1: Wild type 26°C				
+ *Vector*	1.1	1.5	0.15	0.64
+ [*pGPD1-YCK1*]	1.5	2.0	0.14	1.2
+ [*pGPD1-YCK2*]	1.4	1.6	0.15	0.92
Exp. 2: Wild type 26°C				
+ *Vector*	1.5	2.1	0.06	0.93
+ [*pGPD1-YCK1*]	1.5	1.5	0.08	1.1
+ [*pGPD1-YCK2*]	1.5	1.9	0.11	1.1
Figure 2.8B				
mss4^{ts} 37°C				
+ [*CEN MSS4*]	2.2	2.7	0.11	1.2
+ [*CEN MSS4 ^{T312A}*]	2.0	2.5	0.08	1.1
+ [*CEN MSS4 ^{T312D}*]	1.8	2.9	0.11	0.82

Yeast strains incubated at the indicated temperatures were labeled with ^{3}H-*myo*-inositol. Lipids were extracted and deacylated for analysis by HPLC as described. The mean peak area (cpm) of each PIP species is reported as a percentage of the total ^{3}H-labeled lipids.

impaired the growth of cells deficient in PIP 5-phosphatase function (*sjl1*Δ *sjl2*^{ts} *sjl3*Δ *inp54*Δ cells) at a semi-permissive temperature (Figure 2.5B), further suggesting that deletion of the N-terminal region results in increased Mss4 PIP kinase activity. Both Mss4$^{\Delta 726\text{-}779}$-GFP and Mss4$^{\Delta 2\text{-}346}$-GFP were expressed at levels similar to full-length Mss4-GFP, indicating that the altered activities of the mutant proteins were not due to changes in protein stability (Figure 2.3C). In addition, PIK patches containing Mss4-GFP and Mss4$^{\Delta 2\text{-}346}$-GFP displayed similar dynamics and GFP fluorescence intensity distributions (Figure 2.5C and 2.5D). Thus, the increase in PI(4,5)P_2 levels in cells expressing Mss4$^{\Delta 2\text{-}346}$-GFP was not due to increases in Mss4 assembly or PIK patch lifetime.

Regulation of Mss4 PIP kinase activity by potential phosphorylation events

Previous studies demonstrated that Mss4 is a phosphoprotein, and thus the phosphorylation and dephosphorylation cycle may play a role in its PM association (Audhya & Emr, 2003). To test whether this common post-transcriptional modification may also contribute to the regulation of Mss4 kinase activity *in vivo*, I mutated the serine and threonine residues in Mss4's last 54 amino acids, a region that is essential for Mss4 PIK patch assembly and PIP kinase activity (Figure 2.3A and 2.3B). The S/T to A mutation usually mimics the unphosphorylated status of the protein while the S/T to D mutation mimics the phosphorylated form. A growth assay revealed that one serine residue, S728, is a potential phosphorylation site important for Mss4 function, as the Mss4 S728A mutant, but not the S728D mutant, failed to complement the growth of *mss4*^{ts}

cells at the non-permissive temperature. The phosphorylation of this residue is not important for Mss4 PM localization, as we did not detect any change of Mss4 PIK patch assembly of the S728A or the S728D mutant in cells (Figure 2.6A). Instead, the kinase activity is changed as the Mss4 S728A mutant only has residual PI4P-5 kinase activity while the Mss4 S728D mutant could synthesize $PI(4,5)P_2$ at a level comparable to the wild type Mss4 *in vivo* (Figure 2.6B and Table 1).

Another serine residue, S477, was identified as a phosphorylation site by the Smolka lab in a large scale phosphoproteomics study in investigating phosphorylation events in yeast deficient for DNA damage response kinase activity (Marcus Smolka, unpublished data). Both of the Mss4 S477A and Mss4 S477D mutant proteins localize to the PM normally (Figure 2.6A). In contrast to S728, the S477A mutant still retains necessary PIP kinase activity for cell survival; however, the S477D mutant is completely kinase-dead (Figure 2.6C and Table 1). This is consistent with the dephosphin model proposed by Nakano-Kobayashi et al that the several phosphorylation sites in the PIP kinase have to be dephosphorylated to be fully active (Nakano-Kobayashi et al, 2007). Interestingly, we noticed S477 is very close to the ATP-binding of pocket in the Mss4 kinase domain (Figure 1.4). Thus, it is likely phosphorylation at this site might disrupt ATP binding during catalysis to kill the kinase activity of Mss4.

Figure 2.6

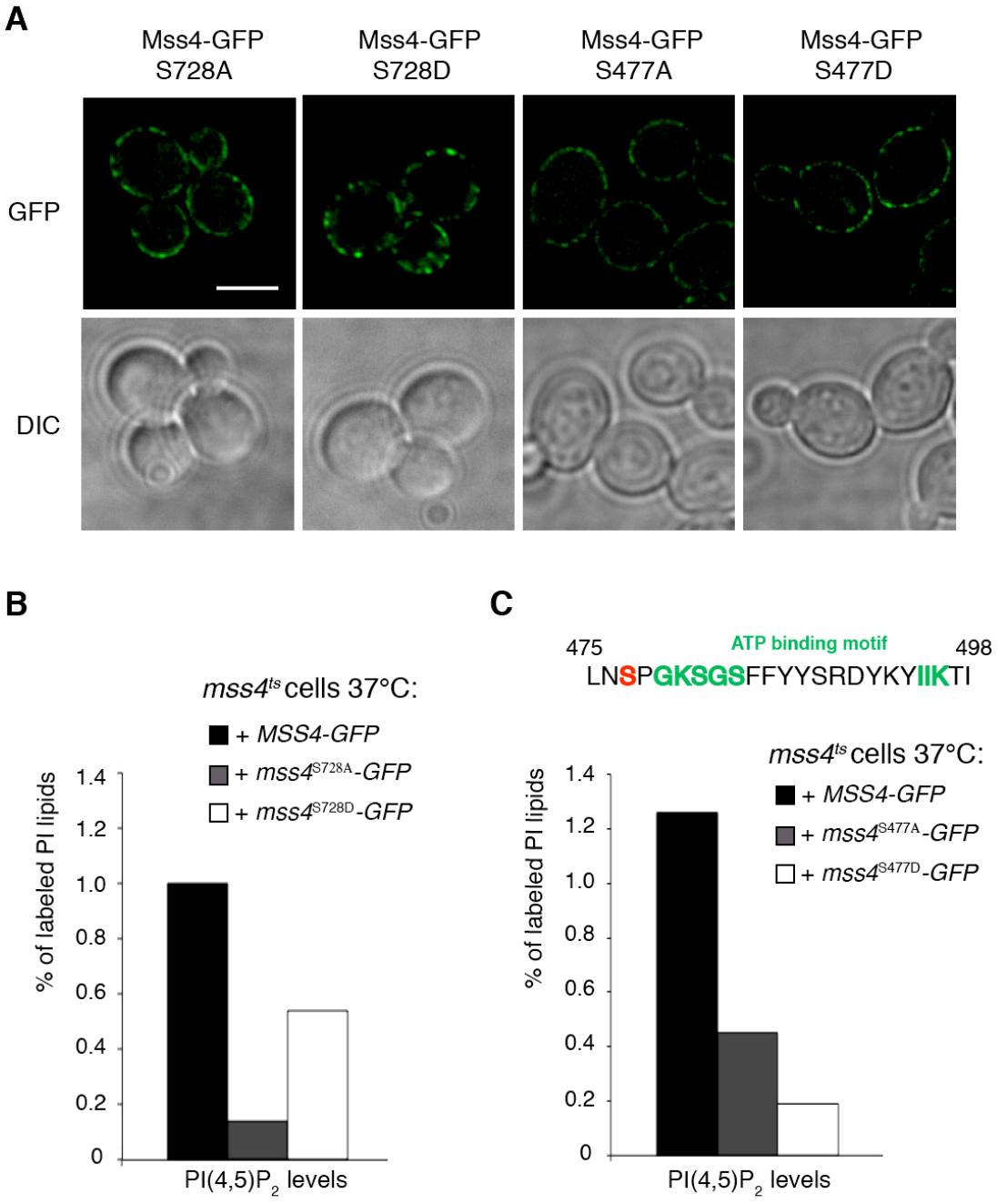

A
Mss4-GFP S728A
Mss4-GFP S728D
Mss4-GFP S477A
Mss4-GFP S477D
GFP
DIC
B
mss4ts cells 37°C:
+ MSS4-GFP
+ mss4S728A-GFP
+ mss4S728D-GFP
% of labeled PI lipids
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
PI(4,5)P2 levels
C
475
ATP binding motif
498
LNSPGKSGSFFYYSRDYKYIIKTI
mss4ts cells 37°C:
+ MSS4-GFP
+ mss4S477A-GFP
+ mss4S477D-GFP
% of labeled PI lipids
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
PI(4,5)P2 levels

Figure 2.6 Potential phosphorylation sites in Mss4 important for its PIP kinase activity

(A) Localization of potential Mss4 phosphorylation site (S728 and S477) mutants. Point mutations were made in the Mss4-GFP construct. Scale bar, 5 μm.

(B) Cellular $PI(4,5)P_2$ levels measured by ^{3}H-inositiol labeling and HPLC analysis of *mss4^{ts}* cells expressing empty vector, Mss4-GFP, Mss4^{S728A}-GFP, or Mss4^{S728D}-GFP. The lipid labeling was performed at 37°C, a non-permissive temperature for the *mss4^{ts}* cells. Results from a representative experiment are shown.

(C) Cellular $PI(4,5)P_2$ levels measured by ^{3}H-inositiol labeling and HPLC analysis of *mss4^{ts}* cells expressing Mss4-GFP, Mss4^{S477A}-GFP, or Mss4^{S477D}-GFP. The lipid labeling was performed at 37°C, a non-permissive temperature for the *mss4^{ts}* cells. Results from a representative experiment are shown.

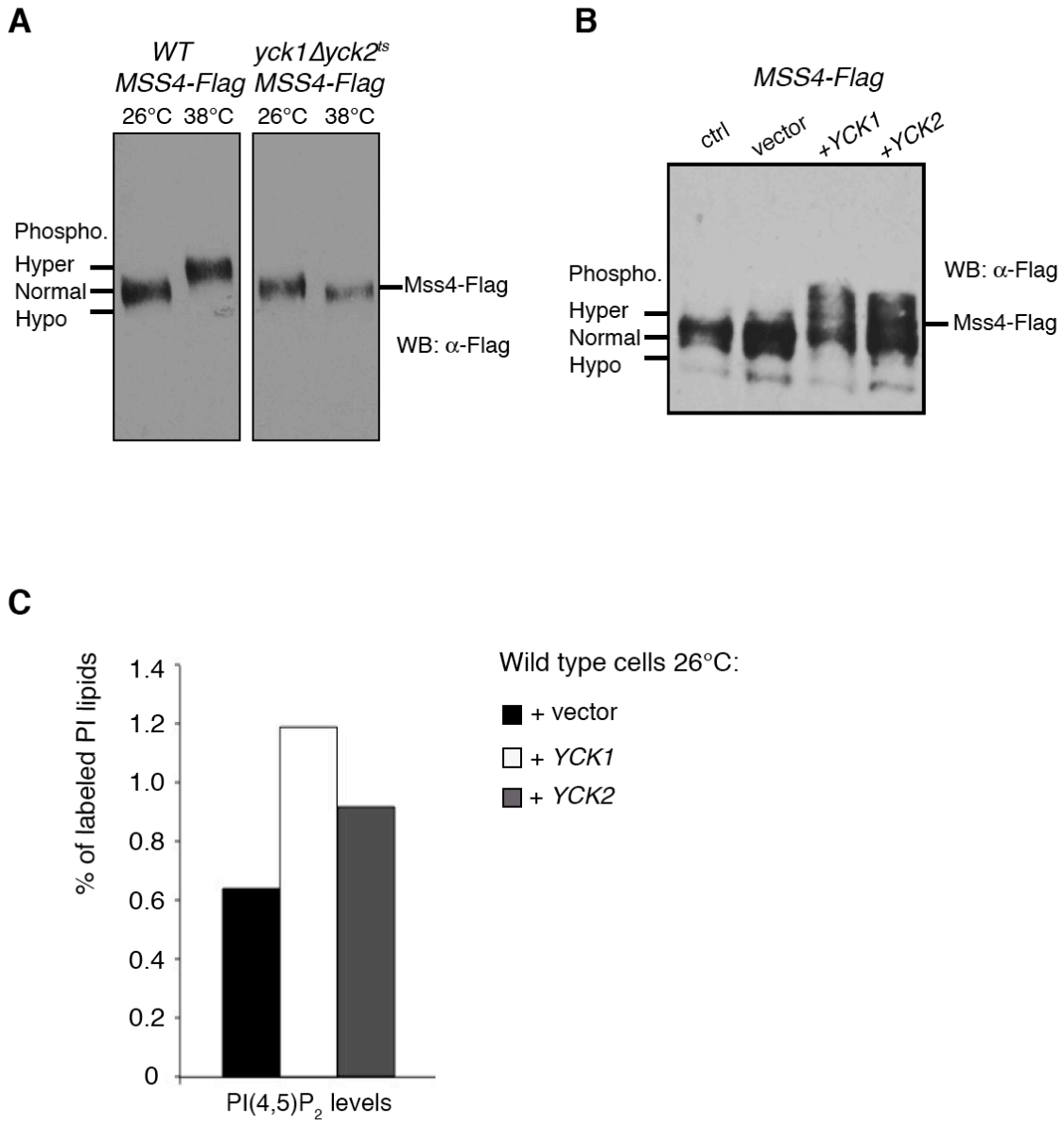
Yck1/2-dependent phosphorylation stimulates Mss4 PIP kinase activity

The Mss4 S728 mutant and S477 mutant results strongly suggest that both protein kinases and phosphatases participate in the activation of Mss4 *in vivo*. Previous studies showed that $PI(4,5)P_2$ levels in the cells are increased upon heat shock (Audhya & Emr, 2002). To examine whether the increase in $PI(4,5)P_2$ correlates with a change in the phosphorylation status of Mss4, I isolated Mss4 from cells grown at 26°C or cells shifted to 38°C for 1 hr. Interestingly, I can clearly detect a band shift of Mss4 purified from cells incubated at 38°C (Figure 2.7A). Previous studies showed that Mss4 could be phosphorylated by the casein kinase I homologs Yck1 and Yck2 both *in vitro* and *in vivo* (Audhya & Emr, 2003; Ptacek et al, 2005), so I tested if this heat-induced band shift of Mss4 is dependent on the kinase activity of Yck1 and Yck2. Interestingly, upon inactivation of Yck1/2 in cells, the heat-induced band shift of Mss4 is no longer detectable at 38°C (Figure 2.7A), indicating that this band shift represents the phosphorylation of Mss4 dependent on Yck1/2. To examine if Yck1 and Yck2-dependent phosphorylation may lead to activation of Mss4, I over-expressed Yck1 or Yck2 in cells expressing Mss4-Flag. Intriguingly, over-expression of Yck1 or Yck2 resulted in a dramatic band shift of Mss4-Flag even at 26°C (Figure 2.7B), further suggesting Mss4 is a Yck1/2 substrate at the PM. To examine if this Yck1/2-dependent phosphorylation of Mss4 may lead to a change in its PIP kinase activity, I performed the lipid labeling experiments to measure the $PI(4,5)P_2$ levels in cells. Consistent with our expectation, over-expression of Yck1 and Yck2 leads to a ~40% and ~25% increase of $PI(4,5)P_2$ levels, respectively

(average of two experiments). Together, these results suggest that Yck1 and Yck2 can phosphorylate Mss4 at the PM and that this phosphorylation could stimulate Mss4 PIP kinase activity *in vivo*.

To identify the Yck1/2 phosphorylation sites in Mss4, we carried out a quantitative mass spec experiment (SILAC-IMAC, for more details, refer to Chapter 3) to examine what residues are hyper-phosphorylated in Mss4 upon over-expressing Yck1. We found that there was ~5 fold increase in phosphorylation on residue T312 in cells over-expressing Yck1 compared to wild type cells (Figure 2.8A). However, neither the phospho-mimetic nor the phospho-defective T312 mutant (T312A or T312D) showed a change in the assembly into PIK patches at the PM (Figure 2.8B), and the PIP kinase activity of both mutants were normal compared to wild type Mss4 *in vivo* as revealed by the lipid labeling experiment (Figure 2.8C and Table 1). So it is likely that additional Yck1/2 phosphorylation sites in Mss4 may exist and contribute to Mss4 PIK patch assembly at the PM. Alternatively, the mis-localization of Mss4 in the *yck1Δyck2*^{ts} mutant cells may be an indirect effect due to the change of phosphorylation status of another Yck1/2 substrate at the PM that is important for PIK patch assembly.

Figure 2.7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WT
MSS4-Flag
yck1Δyck2ts
MSS4-Flag
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Phospho.
Hyper
Normal
Hypo
Mss4-Flag
WB: α-Flag
B
MSS4-Flag
ctrl
vector
+YCK1
+YCK2
Phospho.
Hyper
Normal
Hypo
WB: α-Flag
Mss4-Flag
C
% of labeled PI lipids
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
PI(4,5)P2 levels
Wild type cells 26°C:
+ vector
+ YCK1
+ YCK2

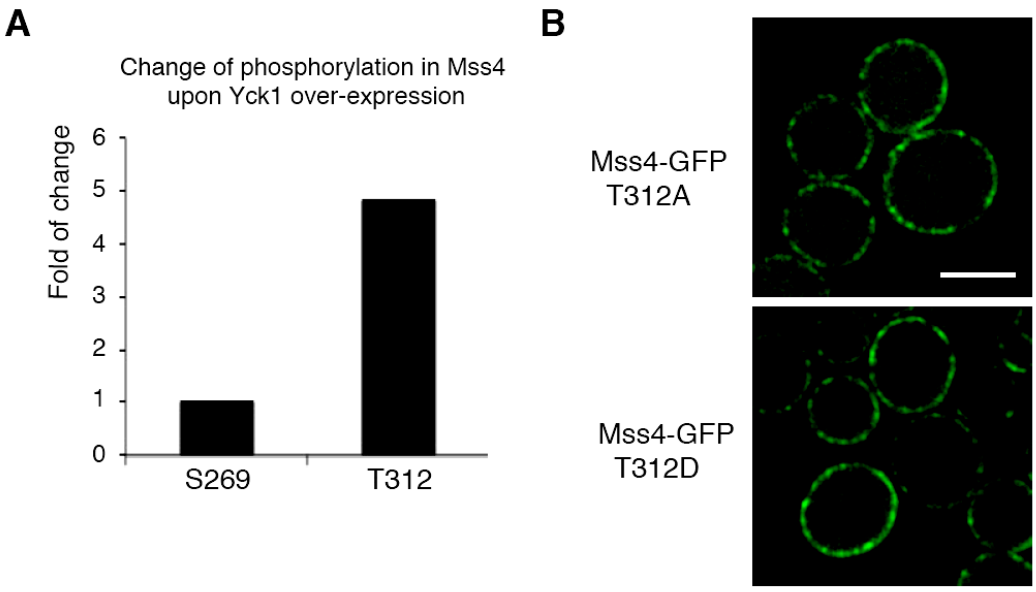
Figure 2.7 Yck1/2-dependent phosphorylation activates Mss4

(A) Mss4 undergoes Yck1/2-dependent phosphorylation upon heat shock. Mss4-Flag was purified from wild type or *yck1*Δ *yck2*^{ts} cells at indicated temperatures and analyzed by SDS-PAGE and western blot.

(B) Over-expression of Yck1 or Yck2 leads to increased Mss4 phosphorylation. Mss4-Flag was purified from wild type cells over-expressing empty vector, *YCK1*, or *YCK2* and analyzed by SDS-PAGE and western blot.

(C) Cellular PI(4,5)P_2 levels measured by ^{3}H-inositiol labeling and HPLC analysis of wild type cells over-expressing empty vector, *YCK1*, or *YCK2*. The lipid labeling was performed at 26°C. Results from a representative experiment are shown. Additional data are provided in Table 1.

Figure 2.8


A
Change of phosphorylation in Mss4
upon Yck1 over-expression
Fold of change
6
5
4
3
2
1
0
S269
T312
B
Mss4-GFP
T312A
Mss4-GFP
T312D

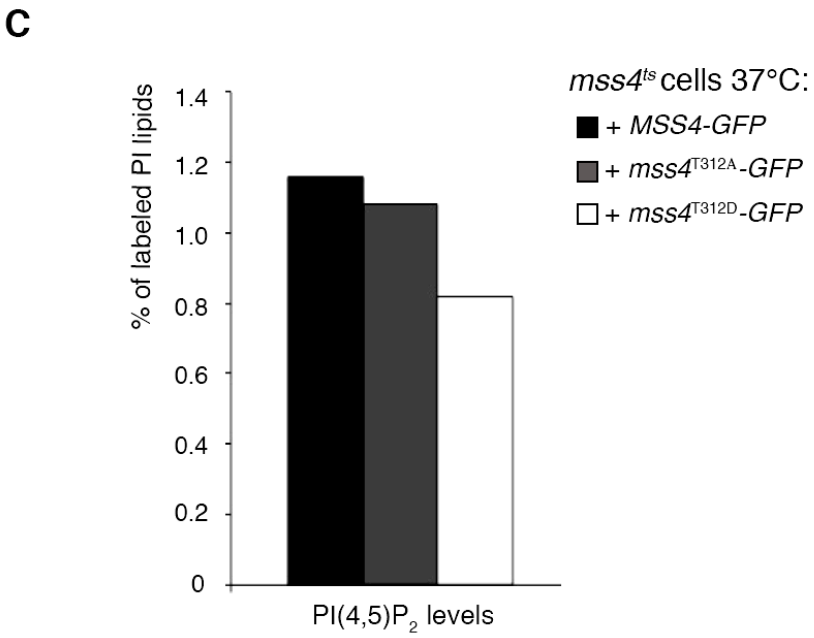

C
% of labeled PI lipids
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
PI(4,5)P2 levels
mss4ts cells 37°C:
+ MSS4-GFP
+ mss4T312A-GFP
+ mss4T312D-GFP

Figure 2.8 The potential Yck1/2 phosphorylation site in Mss4 is not important for PIK patch assembly or PIP kinase activity

(A) SILAC combined with quantitative mass spectrometry analysis was used to measure changes in phosphorylation at specific Mss4 residues for pair wise comparisons of cells over-expressing *YCK1* versus cells expressing empty vector.

(B) Localization of Mss4 T312A and T312D mutants. Point mutations were made in the Mss4-GFP construct. Scale bar, 5 μm.

(C) Cellular $PI(4,5)P_2$ levels measured by ^3H-inositiol labeling and HPLC analysis of *mss4^{ts}* cells expressing Mss4-GFP, Mss4^{T312A}-GFP, or Mss4^{ST312D}-GFP. The lipid labeling was performed at 37°C, a non-permissive temperature for the *mss4^{ts}* cells. Results from a representative experiment are shown.

Discussion

In our study, we showed that the conserved PI4P 5-kinase, Mss4, forms dynamic, oligomeric structures at the plasma membrane that we term PIK patches. The dynamic assembly and disassembly of Mss4 PIK patches may provide a mechanism to precisely modulate Mss4 kinase activity, as needed, for localized regulation of $PI(4,5)P_2$ synthesis.Mss4 PIK patches are distinct from other known cortical structures such as actin patches and eisosomes. The PI 4-kinase Stt4 also oligomerizes at the PM to form PIK patches (Baird et al, 2008). However, Stt4 and Mss4 PIK patches are distinct structures and show unique characteristics (Audhya & Emr, 2002; Baird et al, 2008). Stt4 PIK patches are relatively static at the PM, with a lifetime on the order of three minutes (Baird et al, 2008). Unexpectedly, Mss4 PIK patches are highly dynamic structures undergoing rapid assembly/disassembly and lateral movements. The biological function of PIK patches is not completely clear. However, the assembly of Mss4 into PIK patches may provide spatial and temporal control of $PI(4,5)P_2$ synthesis necessary to coordinate the numerous processes regulated by $PI(4,5)P_2$ (*e.g.* polarized secretion, endocytosis, MAPK activation). Accordingly, individual PIK patches may perform specialized functions and have different compositions (distinct nucleation and regulatory factors). Consistent with this idea, mammalian PI4P 5-kinase isoforms are differentially recruited to focal adhesion sites and clathrin-coated pits by talin and the μ2 AP-2 adaptor subunit, respectively (Krauss et al, 2006; Ling et al, 2002; Nakano-Kobayashi et al, 2007).

The assembly of Mss4 PIK patches involves the activation loop in the Mss4 lipid kinase domain and its substrate PI4P at the PM (Figure 2.9A). Interestingly, we found that Mss4 becomes mis-localized only after inactivation of both Stt4 and Pik1, which are responsible for PI4P generation at the PM and Golgi respectively. This observation suggests that there is still a pool of PI4P at the PM in the *stt4* mutant that could be utilized by Mss4. We think this pool of PI4P is synthesized by Pik1 at the Golgi, and later transported to the PM by Golgi-generated secretory vesicles. Consistent with our hypothesis that Mss4 can use PI4P generated by Stt4 and Pik1 as substrate, inactivation of either of these PI 4-kinases only resulted in a 50% drop of the $PI(4,5)P_2$ level *in vivo* and the PM localization of the $PI(4,5)P_2$ probe GFP-2xPH was not affected (Audhya et al, 2000).

Mss4 PIK patch assembly and PIP kinase activity could be regulated by phosphorylation, as indicated by previous studies (Audhya & Emr, 2003). Here we showed that the phosphorylation at residue S477 has an inhibitory effect on Mss4 PIP kinase activity. Possibly the negative charge present after the phosphorylation event hinders ATP from entering the ATP binding pocket of the Mss4 PIP kinase domain. S728 is another potential phosphorylation site important for Mss4 function. It seems phosphorylation is required at this site, as the S728A has extremely low PIP kinase activity and is not able to synthesize $PI(4,5)P_2$ efficiently to meet the requirement for rapid cell growth. However, we still don't know what kinases/phosphatases are involved in the phosphorylation/

dephosphorylation of these two sites. Consistent with previous observations, we found that the casein kinase I homologs Yck1 and Yck2 could phosphorylate Mss4 *in vivo*, and phosphorylation of Mss4 by Yck1/2 stimulates Mss4 PIP kinase activity (Figure 2.9B). By the mass spectrometry experiment, we found that residue T312 is hyper-phosphorylated upon over-expression of Yck1. However, this residue alone seems not to play an essential role in regulating Mss4 PIK patch assembly or PIP kinase activity. Probably multiple phosphorylation events in Mss4 and possibly in other Yck1/2 substrates may contribute to the increased synthesis of $PI(4,5)P_2$ upon over-expression of Yck1.

Figure 2.9

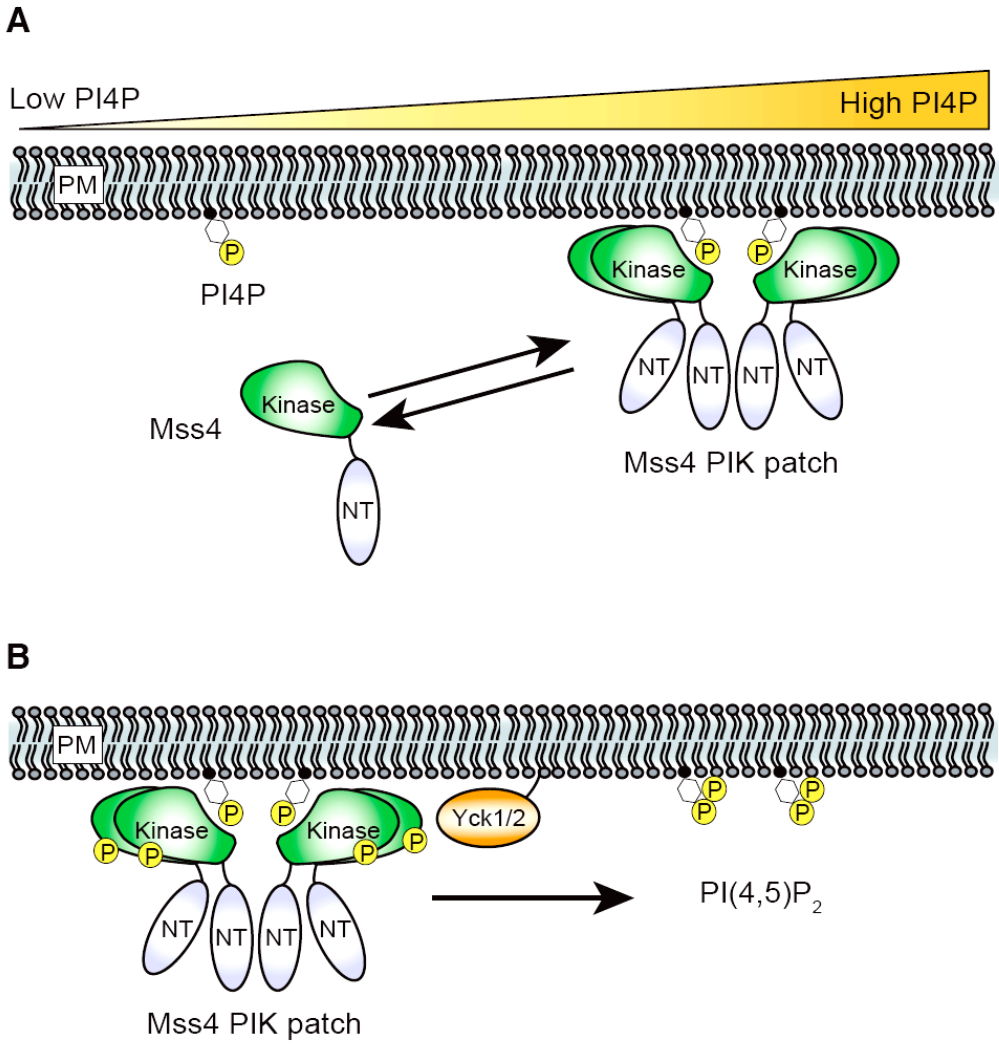

A
Low PI4P
High PI4P
PM
P
PI4P
Kinase
Kinase
NT
NT
NT
NT
Mss4
Kinase
NT
Mss4 PIK patch
B
PM
Kinase
Kinase
Yck1/2
PI(4,5)P2
NT
NT
NT
NT
Mss4 PIK patch

Figure 2.9 Model for Mss4 PIK patch assembly and regulation by phosphorylation at the PM

(A) Above a threshold PI4P concentration, Mss4 is recruited to its substrate at the PM where it oligomerizes and assembles into PIK patches. Localized depletion of PI4P levels by Mss4 PIP kinase activity may promote the disassembly of Mss4 PIK patches. (B) Mss4 is phosphorylated by Yck1 and Yck2 at the PM. Phosphorylation of Mss4 by Yck1 and Yck2 stimulates Mss4 PIP kinase activity. Additional protein kinases may phosphorylate Mss4 that may lead to activation or inhibition of $PI(4,5)P_2$ synthesis.

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Pruyne D, Bretscher A (2000b) Polarization of cell growth in yeast. I. Establishment and maintenance of polarity states. *J Cell Sci* **113 (Pt 3):** 365-375

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Chapter 3

Regulation of PI(4,5)P_2 Synthesis at Mss4 PIK Patches By the Dual PH Domain Protein Opy1[2]

Introduction

The mechanisms of Mss4 regulation at the PM are poorly characterized. Though PI4P is necessary for Mss4 PIK patch assembly, it is not sufficient for recruiting Mss4 to the membrane. We reasoned that the localization and regulation of Mss4 are mediated in part by associated factors. Previous studies indicated that several proteins may control Mss4 PIP kinase activity at PIK patches, including the small calcium-binding protein calmodulin (Desrivieres et al, 2002) and the small GTPase Arf3 (Smaczynska-de et al, 2008). However, it is not known whether these proteins directly bind Mss4 or indirectly regulate Mss4 function. To identify Mss4 interacting partners that regulate Mss4 PIK patch assembly and PIP kinase activity, we carried out a quantitative SILAC-MS proteomic experiment. By this approach, we identified the dual PH domain-containing protein, Opy1, as a novel regulator of Mss4. This chapter will focus mainly on the cellular functions of Opy1 and how it regulates PI(4,5)P_2 levels at the PM.

[2] Some of the experiments presented in this chapter were published in Ling Y. *et al*, *EMBO J*, 2012. The dissertation author was the primary investigator and author of the paper.

Materials and Methods

SILAC and Quantitative Mass Spectrometry Analysis

For quantitative mass spectrometry analysis using SILAC (Stable isotope labeled amino acids in cell culture), yeast strains auxotrophic for lysine and arginine were grown to mid-log phase in the presence of either heavy or light lysine and arginine isotopes. Following bait affinity purification (as described in Materials and Methods in Chapter 2), light and heavy elutes were mixed, reduced with 10mM DTT and alkylated with 20mM iodoacetimide (Sigma-Aldrich). Proteins were then precipitated by adding (3:1) 50% acetone, 49.9% ethanol, and 0.1% acetic acid, resuspended in 8M urea, 50mM Tris-HCl pH=8.0, diluted 3:1 with water and digested with 1µg of trypsin overnight. Tryptic peptides were fractionated by hydrophilic interaction chromatography (HILIC), dried, and reconstituted in 0.1% trifluoroacetic acid. Each fraction was analysed by LC-MS/MS using an Orbitrap XL mass spectrometer (Thermo). Peptide analysis and SILAC quantitation was performed using Sorcerer software. For phosphoprofiling experiments (as described in Chapter 2), phosphopeptides were purified using IMAC chromatography as previously described (Albuquerque et al, 2008). Purified peptides were dried, reconstituted in 0.1% trifluoroacetic acid, and analyzed by LC-MS/MS using an Orbitrap XL mass spectrometer. Database search and SILAC quantitation was performed using Sorcerer software.

Fluorescence Microscopy and Quantification of Fluorescence Intensity

Yeast cells expressing fluorescent fusion proteins were grown to mid-log in synthetic media. Microscopy was performed using a fluorescence microscope (DeltaVision RT; Applied Precision) equipped with FITC and rhodamine filters. Images were captured with a digital camera (Cool Snap HQ; Photometrics) and deconvolved using softWoRx 3.5.0 software (Applied Precision). For comparing PIK patch fluorescence intensity, *opy1*Δ cells and cells overexpressing *OPY1* were grown to mid log phase in YNB media. *opy1*Δ cells were stained with FM4-64, cells overexpressing *OPY1* were stained with Hoechst, and wild type cells were not labeled with a second dye to distinguish between cell types. Cells were then mixed together and examined by fluorescence microscopy in the same field.

Lipid Binding Assays

For liposome flotation assays, 97%PC:3%PI(4,5)P_2 (Avanti Polar Lipids) liposomes were generated by dehydration in a chloroform:methanol mix using a speed vaccuum centrifuge, and then rehydrated in phosphate buffered saline (PBS) with 1 mM DTT at a final concentration of 1.2 mM total lipid. To create unilamellar liposomes, the liposomes were sonicated for 1 minute at room temperature in a water bath. Liposomes (1.2 mM) were then mixed with an equal volume (50 μl) of GST fusion proteins (resulting in 1.7μM final protein and 0.6 mM final lipid concentrations) and incubated for 30 minutes at room temperature. Samples were then resuspended in 200 μl buffer (PBS, 1mM DTT) containing 30% Optiprep density gradient medium (Sigma) and pipetted into thick walled

2.2ml centrifuge tubes. 150μl of 15% Optiprep buffer was overlaid over the samples, followed by 50μL of buffer (PBS, 1mM DTT). Samples were centrifuged for 1 hour at 55,000rpm at 10°C in a TLS-55 ultracentrifuge rotor in desktop ultracentrifuge (Beckman Coulter). The top 200μl of the gradient containing liposomes and bound proteins was then collected as the "bound" fraction. The bottom "unbound" fraction was collected separately. Samples were resuspended in protein sample buffer, run on 10% SDS-PAGE gels, and GST fusion proteins were detected by immuno-blotting using GST antisera. The relative amounts of fusion proteins in bound and unbound fractions were determined using NIH ImageJ analysis software.

For the liposome sedimentation assays, 100%PC, 97%PC:3%PtdIns and 97%PC:3%PIP-containing liposomes were prepared as described above except that the final lipid concentration was 0.3 mM in the binding reactions. Liposomes were incubated with 1.7 μM recombinant GST, GST-Opy1, GST-PH1, or GST-PH2 fusion proteins for 30 minutes at room temperature, and centrifuged at 13,000 x *g* for 20 minutes at 4^{o}C. The resulting supernatant and pellet fractions were prepared for SDS-PAGE analysis and Coomassie-stained to detect recombinant proteins. The gels were scanned using an Odyssey imager (LI-COR) and relative amounts of fusion proteins in pellet (bound) and in supernatant (unbound) fractions were determined using Odyssey analysis software (LI-COR).

Protein Binding Assays

For Mss4-3xHA and GST-Opy1 binding, GST, GST-Opy1, GST-PH1, or GST-PH2 fusion proteins immobilized on glutathione sepharose 4B were incubated with Mss4-3xHA lysates solubilized in Tris IP buffer (50mM Tris-HCl, 150mM NaCl, 5mM EDTA, 0.5% Tween-20, pH=7.5, with protease inhibitors added) for 1 hour at 4°C. Beads were washed with IP buffer three times and elutes were analyzed by SDS-PAGE and immunoblotting using anti-HA antibodies. Similarly, For Mss4 and TAPP1 binding (results shown in Chapter 4), His_6-SUMO-$Mss4^{1-346}$ and His_6-SUMO-$Mss4^{347-779}$ proteins immobilized on Ni-NTA beads were incubated with cell lysates expressing GFP-TAPP1 or GFP alone in Tris IP buffer for 1 hour at 4°C. Beads were washed with IP buffer three times and elutes were analyzed by SDS-PAGE and immunoblotting using GFP antisera.

Mss4-GFP visual screen

Yeast strains from the Tet-promoter Hughes yeast collection (Thermo Fisher Scientific) were transformed with the *MSS4-GFP* plasmid. Yeast cells were grown to mid-log phase in synthetic media without doxycline. Then cells were cut back to 0.05 and grown in synthetic media with 50 μg/mL or without doxycline for 12-18hrs. Cells were then examined by fluorescence microscopy as described above. Results are presented in Chapter 4.

Additional related materials and methods are the same as described in Chapter 2.

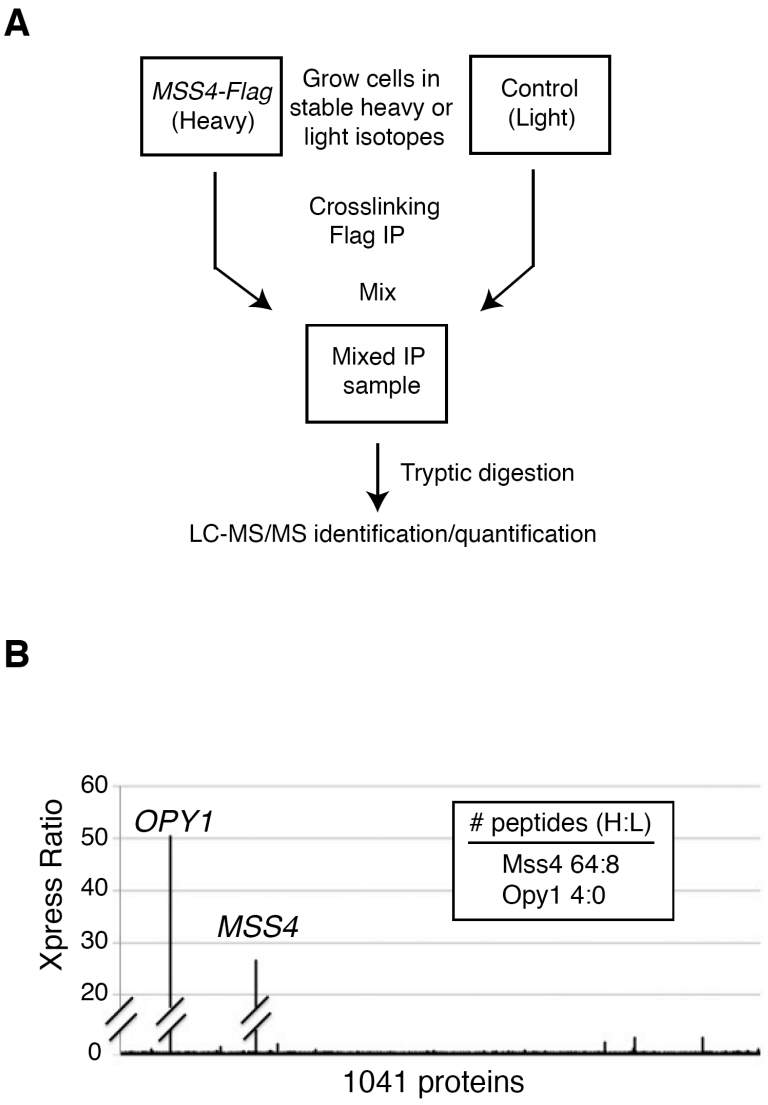
Results

The PH domain-containing protein Opy1 binds Mss4

To identify candidate proteins that interact with Mss4, we undertook a quantitative proteomics approach (Figure 3.1A). Cells were grown in media containing either normal (for the control strain) or heavy isotope amino acids (for cells expressing Mss4-3xFlag). We then performed crosslinking immuno-purification (IP) experiments and processed the protein samples for quantitative mass spectrometry analysis. This approach detects the enrichment of proteins that specifically interact with Mss4, and thus is optimal for the identification of weak/transient interactions. One protein, Opy1, was highly enriched with purified Mss4-3xFlag (Figure 3.1B).

To confirm the Mss4-Opy1 interaction, we repeated the crosslinking-coIP experiment and monitored the results by immunoblotting. As expected, Opy1-3xHA was present in immunoprecipitates from cells expressing Mss4-3xFlag, but not control cells lacking Mss4-3xFlag (Figure 3.1C).

Figure 3.1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MSS4-Flag
(Heavy)
Grow cells in
stable heavy or
light isotopes
Control
(Light)
Crosslinking
Flag IP
Mix
Mixed IP
sample
Tryptic digestion
LC-MS/MS identification/quantification
B
Xpress Ratio
60
50
40
30
20
0
OPY1
MSS4
peptides (H:L)
Mss4 64:8
Opy1 4:0
1041 proteins

Figure 3.1

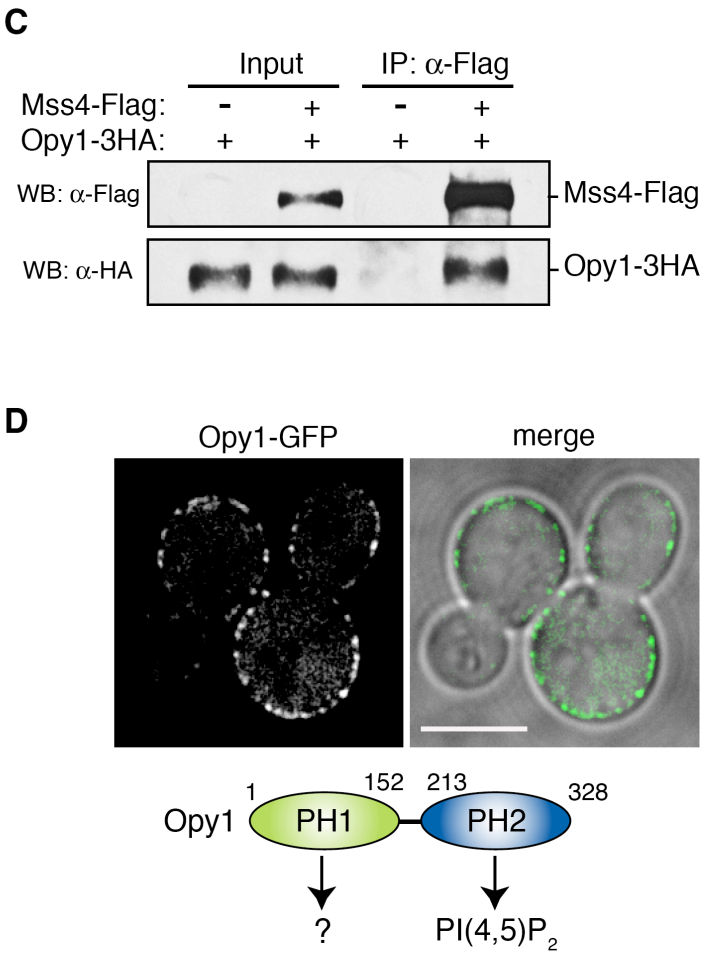

C
Input
IP: α-Flag
Mss4-Flag: - + - +
Opy1-3HA: + + + +
WB: α-Flag
Mss4-Flag
WB: α-HA
Opy1-3HA
D
Opy1-GFP
merge
1
152
213
328
Opy1
PH1
PH2
?
PI(4,5)P2
E
Opy1-GFP
Mss4-RFPmars
merge + DIC
V
V
V
V

Figure 3.1 Identification of Mss4 interacting proteins by chemical crosslinking experiments and quantitative mass spectrometry

(A) Outline of the quantitative SILAC-MS approach. See the Results and Methods for additional details.

(B) Expression ratio (Xpress ratio=Mss4-3xFlag IP/ control IP) for proteins identified by the SILAC-MS experiments. An Xpress ratio of greater than 10 was used a set point to define specific Mss4-interacting proteins. Opy1 was enriched more than 50-fold in the Mss4-3xFlag IP compared to control IP. The inset shows the number of Mss4 and Opy1 peptides identified in the heavy (containing Mss4-3xFlag) and light samples.

(C) Opy1-3HA crosslinks and co-immunoprecipitates with Mss4-Flag. Lysates from cells expressing Opy1-3HA or Mss4-Flag and Opy1-3HA were incubated with crosslinker and incubated with anti-Flag beads. Immunoprecipitates were analyzed by immunoblotting to detect Mss4-Opy1 interactions.

(D) Opy1 localizes to cortical structures. Wild type cells expressing Opy1-GFP were grown to mid-log and examined by fluorescence microscopy. Cells shown are representative of over 100 cells observed. Scale bar, 5 μm. Diagram of the Opy1 protein is shown under the fluorescence images. PH, pleckstrin homology domain.

(E) Localization of Mss4-RFPmars and Opy1-GFP in cells. Arrow heads indicate the co-localization of two proteins. Star indicates regions of Mss4 PIK patches where Opy1-GFP is not present. Scale bar, 1 μm. v, vacuole.

Opy1 consists of two PH domains and the C-terminal PH domain binds $PI(4,5)P_2$ at the PM (Szentpetery et al, 2009; Yu et al, 2004). By examining full-length Opy1-GFP *in vivo*, we observed Opy1 in the cytoplasm and at cortical punctate structures (Figure 3.1D). Interestingly, the cortical Opy1 structures partially co-localized with Mss4 PIK patches at the PM (Figure 3.1E). Taken together, these results suggested that Opy1 interacts with Mss4 at PIK patches.

Opy1 is a novel regulator of $PI(4,5)P_2$ synthesis

To study the function of Opy1 *in vivo*, we first deleted the *OPY1* gene. Mss4 assembled into PIK patches at the PM in cells lacking *OPY1* and thus Opy1 was not essential for Mss4 PIK patch formation (Figure 3.2A). To test if Opy1 regulates $PI(4,5)P_2$ metabolism, we performed ^{3}H-inositol labeling experiments to measure cellular PIP levels. We detected a two-fold increase in $PI(4,5)P_2$ levels in *opy1*Δ cells compared to wild type cells (Figure 3.3A and Table 2). In addition, over-expression of Opy1 in wild type cells resulted in a 40% decrease in $PI(4,5)P_2$ levels (Figure 3.3A and Table 2). Over-expression of Opy1 did not lead to decreases in Mss4 PIK patch lifetime (Figure 3.2B) or affected PIK patch assembly (as measured by relative Mss4-GFP fluorescence intensity, Figure 3.2C). Moreover, over-expression of Opy1 impaired the growth of *mss4^{ts}* cells at a semi-permissive temperature (34^{o}C, Figure 3.3B), further suggesting that Opy1 regulates $PI(4,5)P_2$ metabolism, by either inhibiting its synthesis or by promoting its turnover.

Figure 3.2

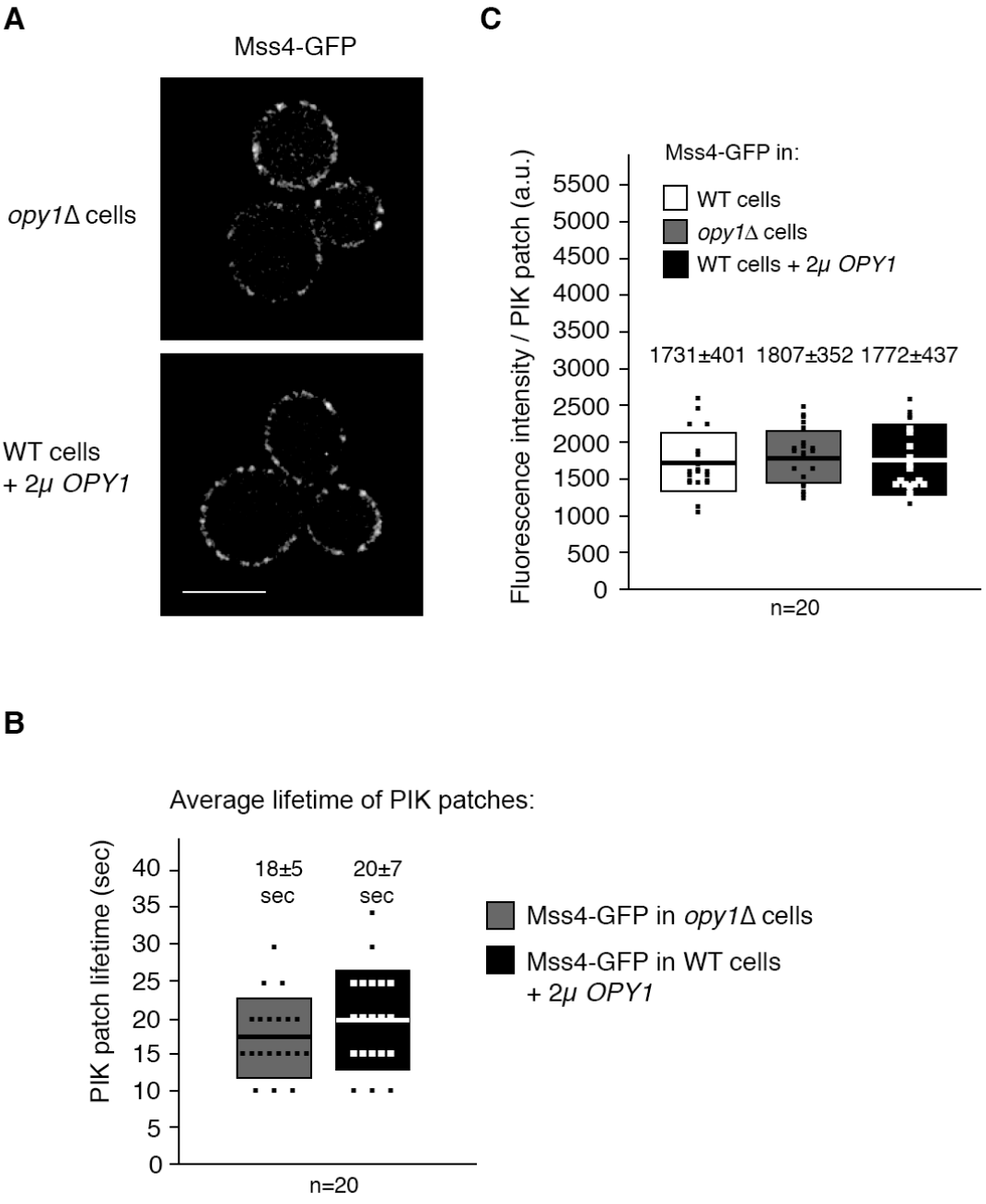

A
Mss4-GFP
opy1Δ cells
WT cells
+ 2μ OPY1
C
Mss4-GFP in:
WT cells
opy1Δ cells
WT cells + 2μ OPY1
1731±401
1807±352
1772±437
Fluorescence intensity / PIK patch (a.u.)
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
n=20
B
Average lifetime of PIK patches:
PIK patch lifetime (sec)
40
35
30
25
20
15
10
5
0
18±5 sec
20±7 sec
n=20
Mss4-GFP in opy1Δ cells
Mss4-GFP in WT cells
+ 2μ OPY1

Figure 3.2 Opy1 does not control Mss4 PIK patch assembly or dynamics

(A) Mss4-GFP localization in *opy1*Δ cells or wild type cells over-expressing *OPY1*. Scale bar, 5 μm.

(B) Measurements of Mss4 PIK patch lifetimes. Mss4-GFP PIK patches in *opy1*Δ cells (dark grey). Mss4-GFP PIK patches in cells overexpressing *OPY1* (black). In each experiment, the lines show mean lifetimes measured for twenty PIK patches (n=20) and boxes indicate standard deviation.

(C) Measurements of Mss4 PIK patch fluorescence intensities. Mss4-GFP PIK patches in wild type cells (white). Mss4-GFP PIK patches in *opy1*Δ cells (dark grey). Mss4-GFP PIK patches in cells overexpressing *OPY1* (black). In each experiment, the lines show mean PIK patch fluorescence intensities measured for twenty PIK patches (n=20) and boxes indicate standard deviation. Endogenous, untagged Mss4 was expressed in all three strains, resulting in reduced Mss4-GFP fluorescence intensities.

Figure 3.3

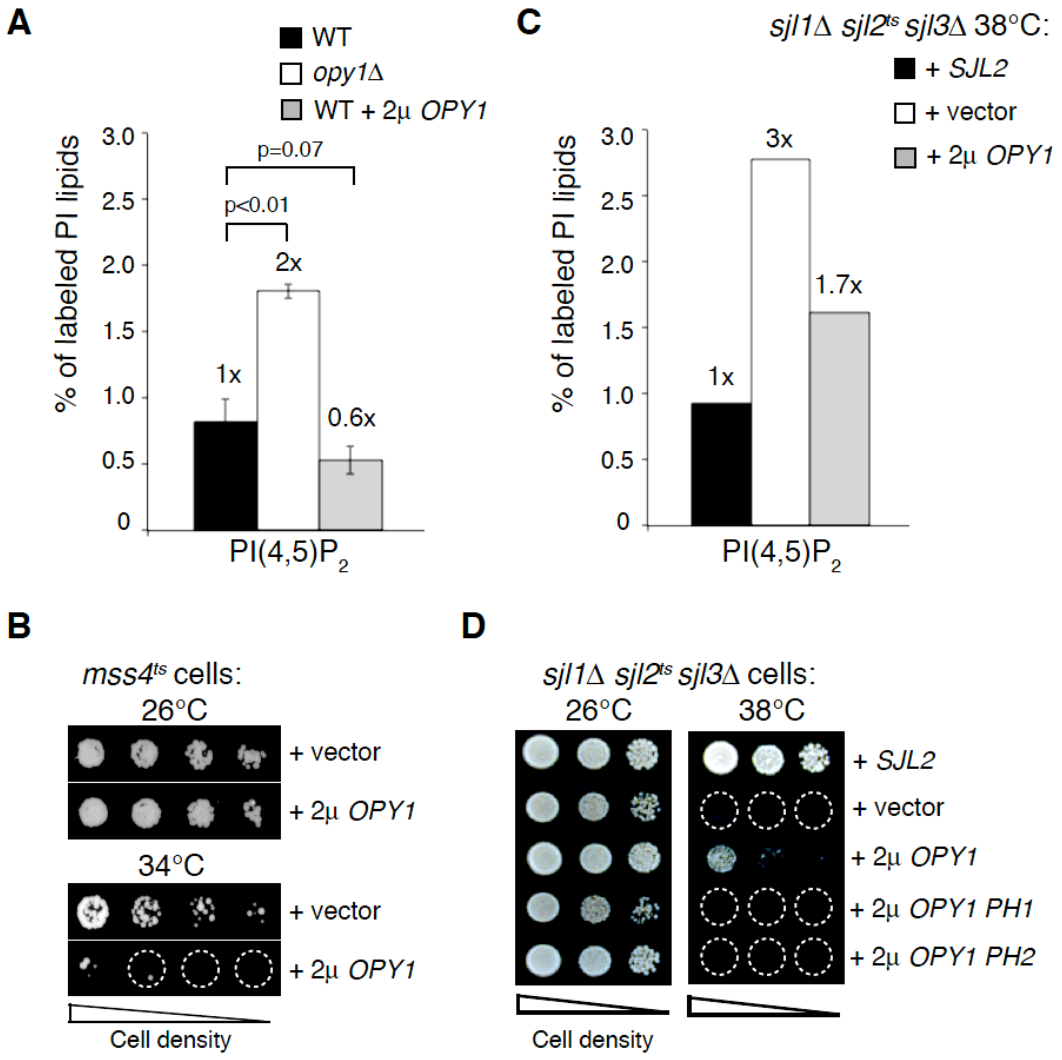
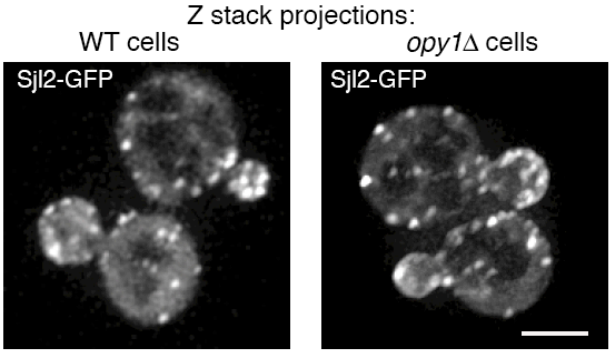
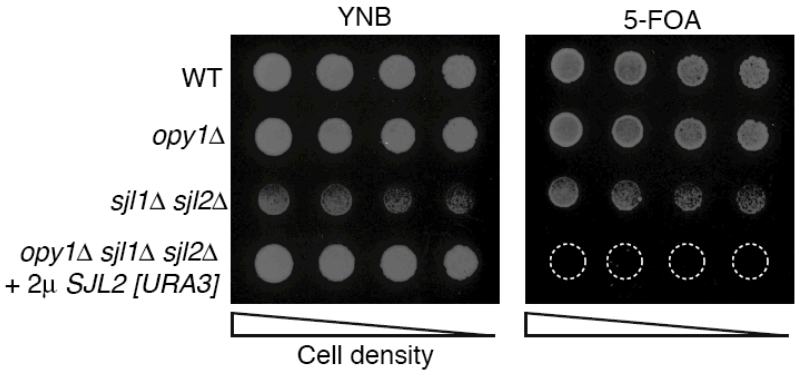

A
WT
opy1Δ
WT + 2μ OPY1
% of labeled PI lipids
3.0
2.5
2.0
1.5
1.0
0.5
0
p=0.07
p<0.01
2x
1x
0.6x
PI(4,5)P2
C
sjl1Δ sjl2ts sjl3Δ 38°C:
+ SJL2
+ vector
+ 2μ OPY1
% of labeled PI lipids
3x
1.7x
1x
PI(4,5)P2
B
mss4ts cells:
26°C
+ vector
+ 2μ OPY1
34°C
+ vector
+ 2μ OPY1
Cell density
D
sjl1Δ sjl2ts sjl3Δ cells:
26°C
38°C
+ SJL2
+ vector
+ 2μ OPY1
+ 2μ OPY1 PH1
+ 2μ OPY1 PH2
Cell density

Figure 3.3

E



F



G

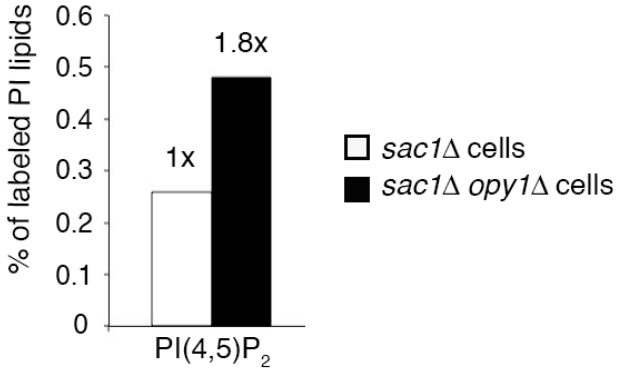


Figure 3.3 Opy1 inhibits PI(4,5)P_2 synthesis at the PM

(A) Cellular PI(4,5)P_2 levels were determined in wild type, *opy1*Δ cells, or wild type cells over-expressing *OPY1* by ^{3}H-inositiol labeling and HPLC analysis. Experiments were performed at 26°C. Data represent the mean of three independent experiments (± standard deviation).

(B) Over-expression of Opy1 impairs the growth of *mss4^{ts}* cells with impaired PIP 5-kinase activity at a semi-permissive temperature (34°C). Serial dilutions of *mss4^{ts}* cells were grown on –Ura plates to retain the high-copy *OPY1* plasmid at either 26°C or 34°C for 3 days.

(C) Cellular PI(4,5)P_2 levels as measured by ^{3}H-inositiol labeling and HPLC analysis in *sjl1*Δ *sjl2^{ts} sjl3*Δ cells carrying empty vector, *SJL2,* or *OPY1* plasmids. Two independent labeling experiments were performed at 38°C. Results from a representative experiment are shown. Additional data are provided in Table 2.

(D) Over-expression of full-length Opy1, but not the PH1 or PH2 domains from Opy1 alone, rescues the growth defect of *sjl1*Δ *sjl2^{ts} sjl3*Δ cells at 38°C. Serial dilutions of *sjl1*Δ *sjl2^{ts} sjl3*Δ cells carrying empty vector, *SJL2*, full-length *OPY1* or truncated *opy1* (PH1 domain or PH2 domain) plasmids as indicated were spotted onto –Ura plates to retain the plasmids and grown at either 26°C or 38°C for 4 days.

(E) Sjl2-GFP localization in wild type or *opy1*Δ cells. Scale bar, 2 μm.

(F) Deletion of *OPY1* impairs the growth of *sjl1*Δ *sjl2*Δ cells with impaired PIP phosphatase activity. Serial dilutions of cells were grown on YNB plates or 5-FOA plates to select for loss of the *URA3*-marked *SJL2* plasmid at 26°C for 3 days.

(G) Cellular PI(4,5)P_2 levels were determined in *sac1*Δ and *sac1*Δ *opy1*Δ cells by ^{3}H-inositiol labeling and HPLC analysis. Results from a representative experiment are shown. Additional data are provided in Table 2.

In an independent genetic screen to identify regulators of $PI(4,5)P_2$ signaling, we isolated *OPY1* as a high copy suppressor of synaptojanin mutant cells deficient in PIP 5-phosphatase activity (Stefan et al, 2002). Consistent with this, *OPY1* over-expression resulted in decreased $PI(4,5)P_2$ levels in *sjl1*Δ *sjl2*^{ts} *sjl3*Δ cells at the restrictive temperature (Figure 3.3C and Table 2). Thus, Opy1 modulates $PI(4,5)P_2$ metabolism independently of the synaptojanin-like PIP phosphatases. Consistent with this, loss of Opy1 did not alter the cortical localization of Sjl2 (Figure 3.3E). Furthermore, triple deletion of *SJL1*, *SJL2*, and *OPY1* resulted in an additive growth defect, as *sjl1*Δ *sjl2*Δ *opy1*Δ cells failed to grow upon loss of a *URA3*-marked *SJL2* plasmid on 5-FOA media (Figure 3.3F), suggesting that Opy1 and the synaptojanins regulate $PI(4,5)P_2$ by distinct mechanisms. We observed that PI4P levels were slightly elevated (1.5-fold) in cells lacking Opy1 (*opy1*Δ cells; Table 2). It was therefore possible that Opy1 could activate a PIP 4-phosphatase such as the Sac1 enzyme (Foti et al, 2001; Stefan et al, 2011), and thus loss of Opy1 could lead to elevated levels of both PI4P and $PI(4,5)P_2$. However, $PI(4,5)P_2$ levels were increased (1.8-fold) in *sac1*Δ *opy1*Δ double mutant cells, as compared to *sac1*Δ single mutant cells (Figure 3.3G and Table 2). Thus, coupled with our co-IP and co-localization results, we propose that Opy1 may inhibit Mss4 activity at PIK patches rather than activate a PIP phosphatase, such as the synaptojanins or Sac1 (Figure 3.8A).

Opy1 is a coincidence detector of PI(4,5)P_2 and Mss4

As Opy1 consists of two PH domains, we next addressed how Opy1 localizes to PIK patches at the PM. The N-terminal PH domain of Opy1 fused to GFP (GFP-PH1) did not localize to the PM *in vivo* (Figure 3.4A; Yu et al, 2004). In contrast, the C-terminal PH domain fused to GFP (GFP-PH2) was sufficient for PM targeting (Yu et al, 2004). Similar to a previous study, the GFP-PH2 fusion still localized to the PM in *mss4^{ts}* cells at the non-permissive temperature (Yu et al, 2004; our unpublished observations). However, we found that GFP-PH2 became mis-localized from the PM to the cytoplasm in *pik1^{ts} stt4^{ts}* double mutant cells upon an extended shift to 37^{o}C (Figure 3.4A). A previous study reported that GFP-PH2 localized to the PM in *pik1^{ts} stt4^{ts}* mutant cells (Yu et al, 2004). However, GFP-PH2 was over-expressed from a high copy plasmid in this study. In addition, Yu et al. found that over-expression of the PH2 domain caused growth defects in *pik1^{ts} stt4^{ts}* cells, suggesting that the PH2 domain may compete for some factor that becomes limiting in these cells. As *pik1^{ts} stt4^{ts}* double mutant cells have significantly reduced levels of PI4P, PI(4,5)P_2, and Mss4 PIK patches at the PM (Audhya et al, 2000), we further addressed the lipid and protein binding activities for full-length Opy1 and the Opy1 PH domains.

First, we examined whether Opy1 bound PI(4,5)P_2-containing liposomes *in vitro*. For these experiments, we used GST, GST-PH1, GST-PH2, and GST-Opy1 fusion proteins purified from bacteria. GST-Opy1 (52% of the total protein) efficiently bound and floated with PC:PI(4,5)P_2-containing liposomes (3% mol

PI(4,5P)$_2$) on an equilibrium density gradient (Figure 3.4B). Likewise, GST-PH2 (41% of the total protein) was present with liposomes following equilibrium density fractionation, suggesting that the PH2 domain was sufficient for lipid binding (Figure 3.4B). In control experiments employing liposome sedimentation assays, neither GST-Opy1 nor GST-PH2 bound liposomes lacking PI(4,5)P_2 (*e.g.* PC:PtdIns-containing liposomes; Figure 3.4C). GST alone or the GST-PH1 fusion did not efficiently bind liposomes (Figure 3.4C and 3.4D).

Next, we examined the PIP-binding specificities of Opy1 and the PH2 domain in liposome sedimentation experiments. GST-Opy1 (32% of the total protein) specifically bound to liposomes containing 3% PI(4,5)P_2, but not to liposomes containing equal concentrations of other PIP isoforms (PI3P, PI4P, or PI(3,5)P_2; Figure 3.4D). The GST-PH2 fusion sedimented with PI4P- and PI(4,5)P_2-containing liposomes (18% and 30% of the total protein, respectively; Figure 3.4D), but not with PI3P- or PI(3,5)P_2-containing liposomes (2% and 1% of the total protein, respectively; Figure 3.4D). As expected, GST alone or the GST-PH1 fusion did not efficiently sediment with PIP-containing liposomes (Figure 3.4C and 3.4D). PI4P binding by the PH2 domain may have resulted in the Pik1- and Stt4-dependent PM localization of the GFP-PH2 fusion *in vivo* (Figure 3.4A). However, unlike the PH2 domain alone, full-length Opy1 specifically bound PI(4,5)P_2 *in vitro*. To confirm whether full-length Opy1 localizes to the PM in a PI(4,5)P_2-dependent fashion, we examined GFP-Opy1 localization in *mss4*^{ts} cells that have reduced levels of PI(4,5)P_2 at the restrictive temperature (Stefan et al,

2002). The PM localization of full-length Opy1-GFP was reduced in *mss4^{ts}* cells shifted to the restrictive temperature but not in wild type cells (Figure 3.4E), suggesting that the recruitment of Opy1 to Mss4 PIK patches was dependent on PI(4,5)P_2 synthesis.

Table 2. Phosphoinositide levels in wild type and mutant cells examined				
Strain	**PIP levels (% of total ^{3}H-labeled PtdIns and PIPs)**			
	PI3P	**PI4P**	**PI(3,5)P_2**	**PI(4,5)P_2**
Fig. 3.3A and 3.5C (n=3)				
Wild type 26°C	1.6±0.31	1.8±0.22	0.07±0.03	0.82±0.17
opy1Δ 26°C	2.1±0.39	2.8±0.16	0.15±0.05	1.8±0.06
Wild type + [*2μ OPY1*] 26°C	1.4±0.28	2.1±0.35	0.05±0.01	0.53±0.11
Fig. 3.3C and 3.6C (n=2)				
Exp. 1 *sjl1Δ sjl2^{ts} sjl3Δ* 38°C:				
+ [*CEN SJL2*]	1.1	1.0	0.15	0.93
+ *Vector*	1.0	1.7	0.69	2.8
+ [*2μ OPY1*]	0.85	1.2	0.32	1.6
+ [*TAPP1*]	0.73	1.2	0.36	1.3
Exp. 2 *sjl1Δ sjl2^{ts} sjl3Δ* 38°C:				
+ [*CEN SJL2*]	1.3	1.8	0.12	1.2
+ *Vector*	0.60	1.3	0.42	3.1
+ [*2μ OPY1*]	0.56	1.3	0.43	1.7
+ [*TAPP1*]	0.88	0.99	0.26	1.0
Fig. 3.3G (n=2)				
Exp. 1:				
sac1Δ 26°C	2.2	22	0.08	0.26
sac1Δ opy1Δ 26°C	1.8	26	0.05	0.48
Exp. 2:				
sac1Δ 26°C	1.6	17	0.06	0.22
sac1Δ opy1Δ 26°C	1.2	28	0.04	0.34
Fig. 3.5C (n=3)				
mss4Δ +CEN MSS4 $^{Δ2-346}$ 26°C				
+ *Vector*	1.7±0.48	1.9±0.39	0.13±0.05	1.2±0.27
+ [*2μ OPY1*]	1.7±0.37	2.1±0.22	0.12±0.05	1.2±0.28

Yeast strains incubated at the indicated temperatures were labeled with ^{3}H-*myo*-inositol. Lipids were extracted and deacylated for analysis by HPLC as described. The mean peak area (cpm) of each PIP species is reported as a percentage of the total ^{3}H-labeled lipids. The number of independent labeling experiments performed is indicated (n). Mean values (± standard deviation) are shown when n=3. Individual measured values are reported when n=2.

Figure 3.4

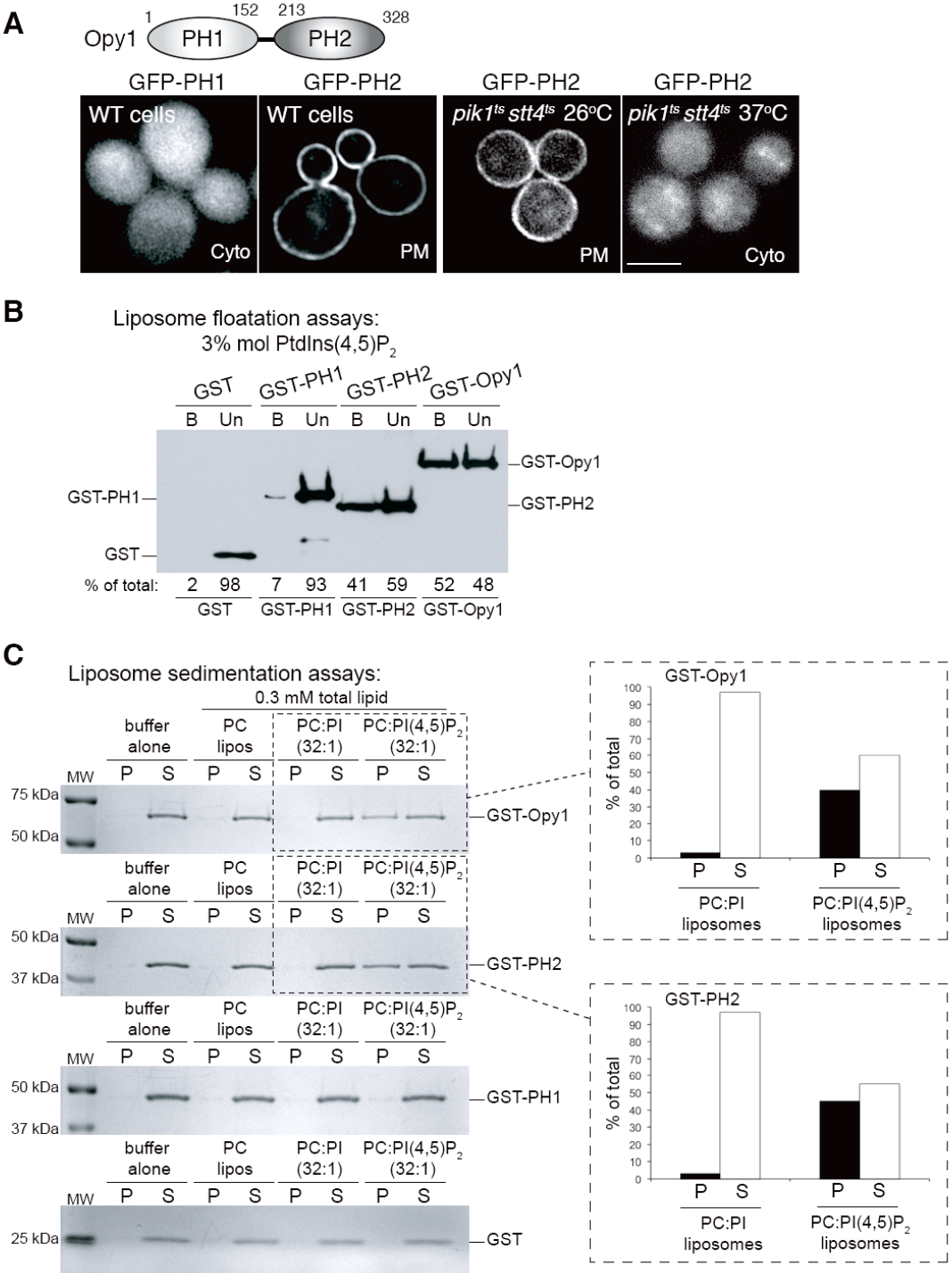

A
Opy1
1
152
213
328
PH1
PH2
GFP-PH1
GFP-PH2
GFP-PH2
GFP-PH2
WT cells
Cyto
WT cells
PM
pik1ts stt4ts 26°C
PM
pik1ts stt4ts 37°C
Cyto
B
Liposome floatation assays:
3% mol PtdIns(4,5)P2
GST
GST-PH1
GST-PH2
GST-Opy1
B Un B Un B Un B Un
GST-Opy1
GST-PH2
GST-PH1
GST
% of total: 2 98 7 93 41 59 52 48
GST
GST-PH1
GST-PH2
GST-Opy1
C
Liposome sedimentation assays:
0.3 mM total lipid
buffer alone
PC lipos
PC:PI (32:1)
PC:PI(4,5)P2 (32:1)
MW
P S P S P S P S
75 kDa
50 kDa
GST-Opy1
50 kDa
37 kDa
GST-PH2
50 kDa
37 kDa
GST-PH1
25 kDa
GST
GST-Opy1
% of total
P S P S
PC:PI liposomes
PC:PI(4,5)P2 liposomes
GST-PH2
% of total
P S P S
PC:PI liposomes
PC:PI(4,5)P2 liposomes

Figure 3.4

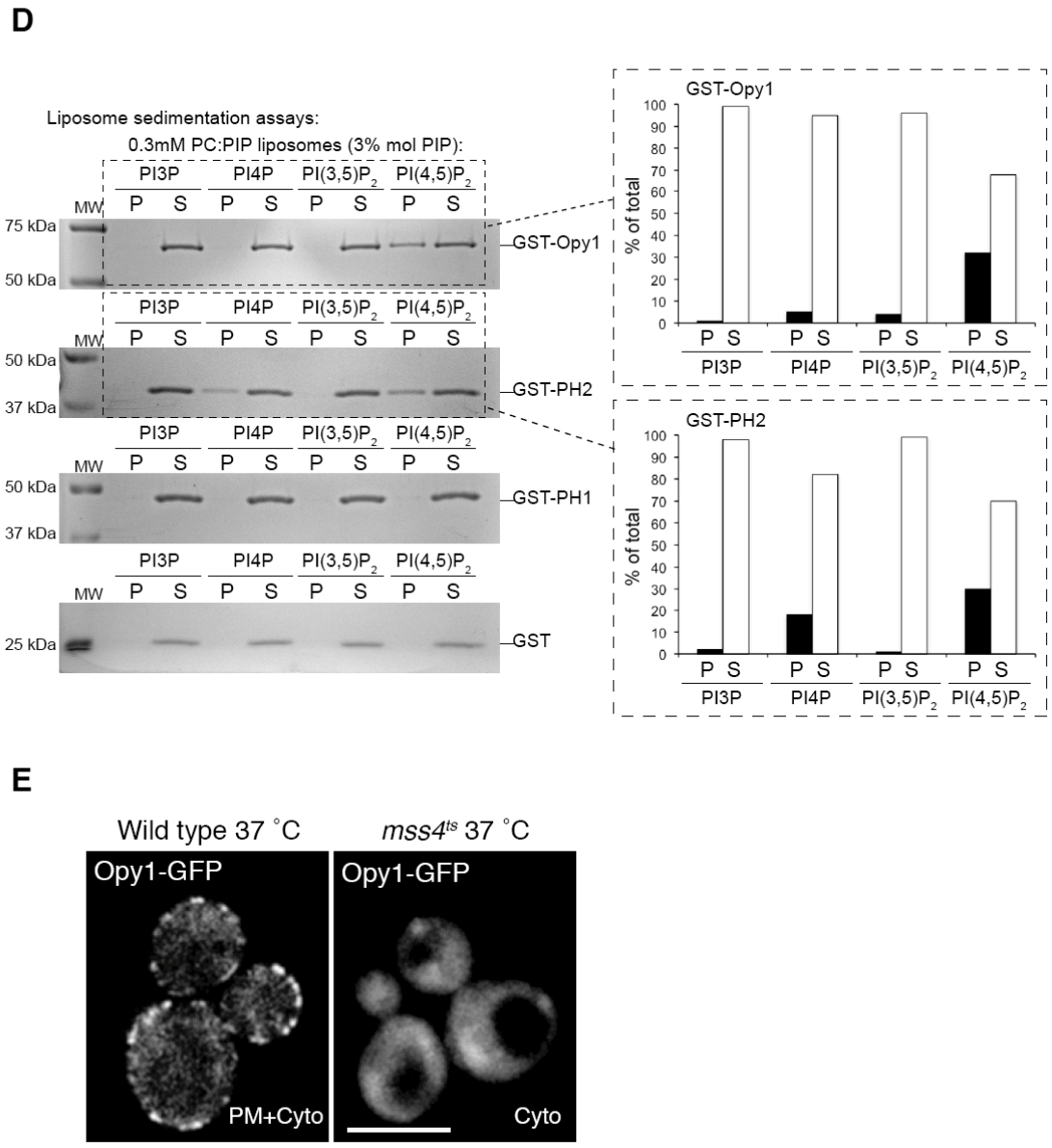


Figure 3.4 The full-length Opy1 and its PH2 domain bind to PI(4,5)P_2

(A) Left panels: Localization of GFP-tagged Opy1 PH1 or PH2 domains in wild type cells. Wild type cells expressing GFP-PH1 or GFP-PH2 were grown to mid-log and examined by fluorescence microscopy. Right panels: Localization of GFP-tagged Opy1 PH2 domain in cells with impaired PtdIns 4-kinase activity. Double mutant *pik1^{ts} stt4^{ts}*

cells expressing GFP-PH2 were grown to mid-log at the permissive temperature (26^{o}C) and shifted to 37°C for 60 minutes, and examined by fluorescence microscopy. Scale bar, 3 μm. Cyto, cytoplasm; PM, plasma membrane.

(B) Full-length Opy1 and the Opy1 PH2 domain bind PI(4,5)P_2-containing liposomes *in vitro*. Recombinant GST, GST-PH1, GST-PH2, and GST-Opy1 fusion proteins were expressed, purified from bacteria, and incubated with PC:PI(4,5)P_2-containing liposomes (0.3 mM total lipid, 3% mol PI(4,5)P_2). Liposomes were separated from unbound protein by floatation on Nycodenz equilibrium gradients (see Materials and Methods). GST fusion proteins in unbound (Un) and liposome-bound (B) fractions were detected by immuno-blotting with GST antisera.

(C) Full-length GST-Opy1 and GST-PH2 fusion proteins specifically bind to PI(4,5)P_2-containing liposomes (97%PC:3%PI(4,5)P_2) in liposome sedimentation assays. Full-length GST-Opy1 and GST-PH2 fusion proteins do not sediment with liposomes lacking PI(4,5)P_2 (e.g. 100%PC or 97%PC:3%PtdIns liposomes). Quantification of GST-Opy1 and GST-PH2 binding to PtdIns-containing and PI(4,5)P_2-containing liposomes is shown (right panels). GST alone and GST-PH1 do not sediment with PI(4,5)P_2 containing liposomes.

(D) Full-length GST-Opy1 specifically sediments with PI(4,5)P_2-containing liposomes, but not with liposomes containing PI3P, PI4P, or PI(3,5)P_2. GST-PH2 binds to both PI4P- and PI(4,5)P_2-containing liposomes. GST alone and GST-PH1 do not sediment with PIP-containing liposomes. Liposomes consisted of 97%PC:3%PIP species. Quantification of GST-Opy1 and GST-PH2 binding to PIP-containing liposomes is shown (right panels).

(E) Opy1 PM targeting is dependent on PI(4,5)P_2 synthesis. Opy1-GFP localization in wild type and *mss4*^{ts} cells incubated at 37°C. Cells were grown at 26°C to mid-log phase, shifted to 37°C for 60 minutes, and examined by fluorescence microscopy. Scale bar, 5 μm. Cyto, cytoplasm; PM, plasma membrane.

Since the Mss4 N-terminal domain attenuates PI(4,5)P_2 synthesis (Figure 2.5A), we addressed whether it regulates Opy1 function. Strikingly, Opy1-GFP localized diffusely in the cytoplasm in cells expressing only the N-terminal truncated form of Mss4 (*mss4*$^{\Delta 2\text{-}346}$ cells, Figure 3.5A). To test if the Mss4 N-terminal region was necessary for the Mss4-Opy1 interaction, we performed crosslinking co-IP experiments using either full-length or N-terminally truncated Mss4. As in our previous results (Figure 3.1C), Opy1 co-purified with full-length Mss4 (Figure 3.5B). However, Opy1 did not interact with the mutant form of Mss4 lacking its N-terminal region (Figure 3.5B), even though this truncated Mss4 localized to the PM and was functional (Figure 2.3A and 2.3B). Therefore, the Mss4 N-terminal region may serve as a scaffold for Opy1 recruitment to PIK patches. Consistent with this, over-expression of *OPY1* in *mss4*$^{\Delta 2\text{-}346}$ cells did not result in a reduction in PI(4,5)P_2 levels (Figure 3.5C and Table 2). Together, these results suggest that the tandem PH domain protein Opy1 serves dual roles as a co-incidence detector of PI(4,5)P_2 and Mss4 as well as a regulator of PI(4,5)P_2 synthesis at the PM.

To further dissect the functions of the two PH domains in Opy1, we over-expressed either the PH1 domain or the PH2 domain in *sjl1*Δ *sjl2*^{ts} *sjl3*Δ cells. Neither of the PH domains was sufficient to complement the growth defect of *sjl1*Δ *sjl2*^{ts} *sjl3*Δ cells at the non-permissive temperature (Figure 3.3D), suggesting they may function together to regulate PI(4,5)P_2 synthesis. However,

to test whether the PH1 domain was functional when artificially targeted to the PM, we fused a palmitoylation motif (from Psr1) to the N-terminus of the PH1 domain, which resulted in its efficient targeting to the PM (Figure 3.6A). Interestingly, this fusion partially rescued the growth defect of *sjl1*Δ *sjl2*^{ts} *sjl3*Δ cells (Figure 3.6B). These results predicted that both PH1 and PH2 might interact independently with Mss4 and have distinct roles in PIK patch targeting and Mss4 PIP 5-kinase regulation. To test this idea, cell lysates containing Mss4-3xHA were incubated with GST, GST-PH1, GST-PH2, or GST-Opy1 fusion proteins bound to glutathione sepharose beads. As expected, full-length GST-Opy1 pulled down Mss4-3xHA from cell lysates but GST alone did not (Figure 3.6C) Interestingly, Mss4-3xHA was isolated with both the GST-PH1 and GST-PH2 fusion proteins in pull-down experiments (Figure 3.6C). Thus, the PH2 domain may target Opy1 to the PM by binding PI(4,5)P_2 and Mss4, and the PH1 domain may serve as a negative regulator of PI(4,5)P_2 synthesis.

Figure 3.5

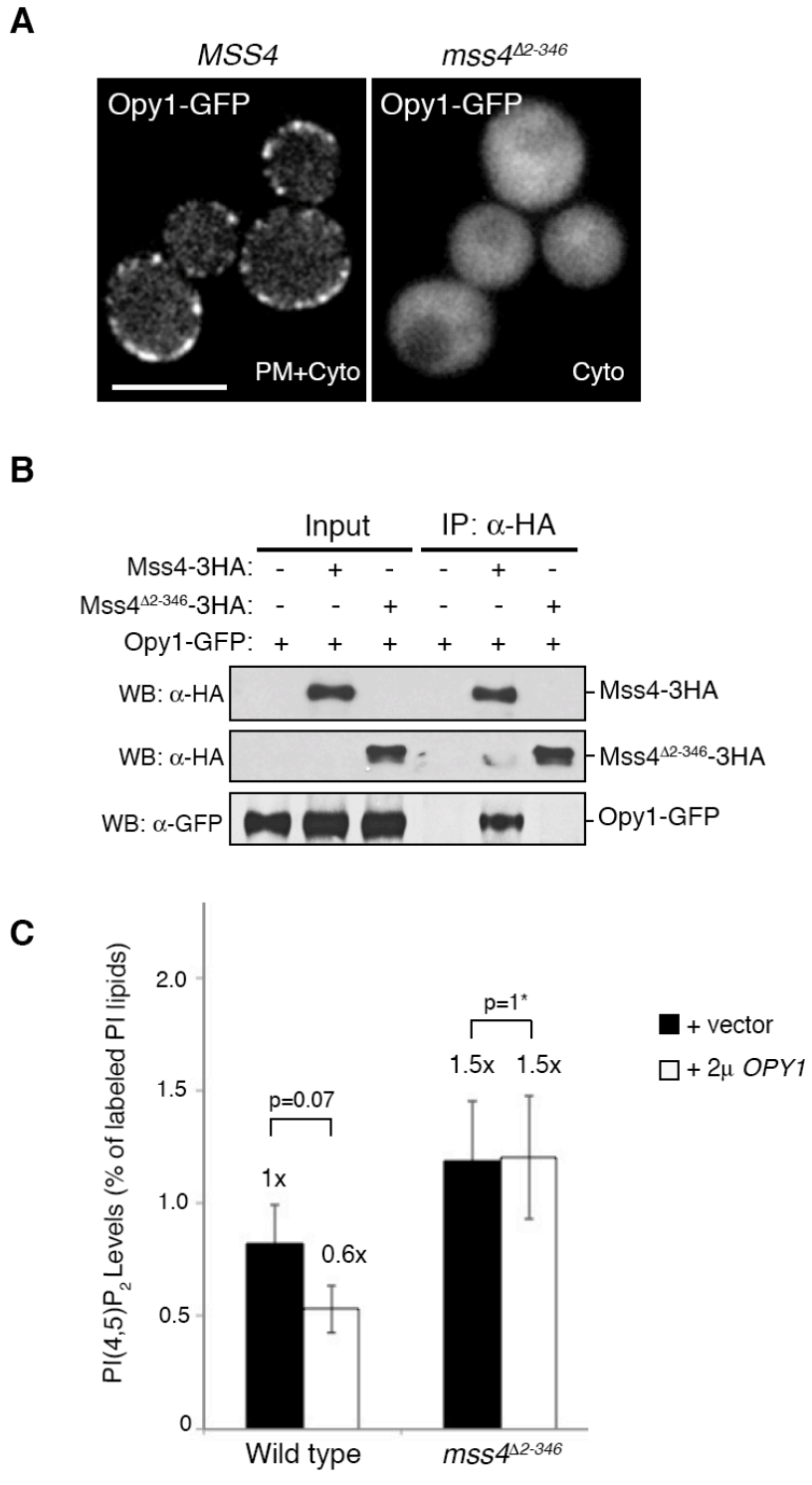

A
MSS4
mss4Δ2-346
Opy1-GFP
Opy1-GFP
PM+Cyto
Cyto
B
Input
IP: α-HA
Mss4-3HA: - + - - + -
Mss4Δ2-346-3HA: - - + - - +
Opy1-GFP: + + + + + +
WB: α-HA
Mss4-3HA
WB: α-HA
Mss4Δ2-346-3HA
WB: α-GFP
Opy1-GFP
C
PI(4,5)P2 Levels (% of labeled PI lipids)
2.0
1.5
1.0
0.5
0
p=0.07
1x
0.6x
p=1*
1.5x
1.5x
+ vector
+ 2μ OPY1
Wild type
mss4Δ2-346

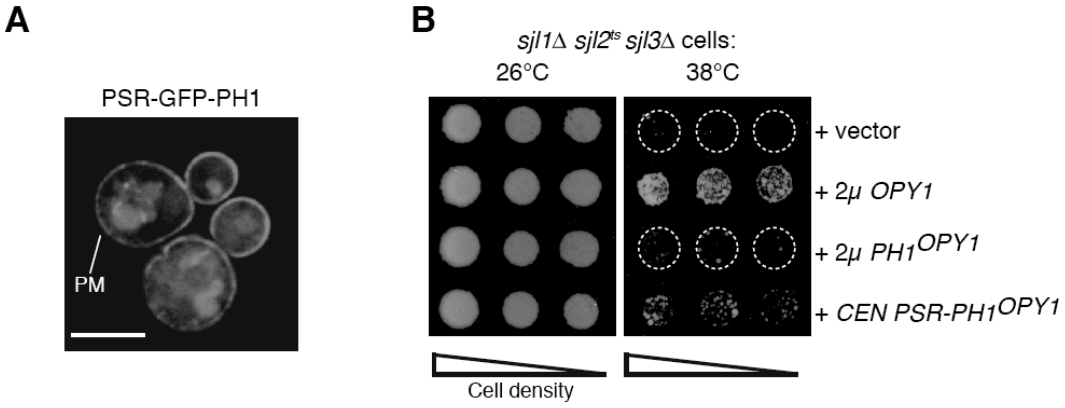
Figure 3.5 Opy1 PM targeting and function is dependent on the Mss4 N-terminus

(A) Opy1-GFP localization in *mss4*Δ cells expressing either full-length Mss4 or the N-terminally truncated Mss4. Scale bar, 5 μm. Cyto, cytoplasm; PM, plasma membrane.

(B) Opy1-GFP crosslinks and co-immunoprecipitates with Mss4-3HA but not with N-terminally truncated Mss4-3HA (lacking residues 2-346). Lysates from cells expressing Opy1-GFP, Opy1-GFP and Mss4-3HA, or Opy1-GFP and $Mss4^{\Delta 2\text{-}346}$-3HA were incubated with crosslinker and incubated with anti-HA beads. Immunoprecipitates were analyzed by immunoblotting to detect Mss4-Opy1 interactions.

(C) Cellular $PI(4,5)P_2$ levels measured by ^{3}H-inositiol labeling and HPLC analysis for wild type cells and $mss4^{\Delta 2\text{-}346}$ cells expressing only the N-terminally truncated form Mss4 carrying a high copy *OPY1* plasmid or vector alone. Three independent labeling experiments were performed at 26°C. Data represent the mean (±standard deviation). *p=1 indicates that there is no statistical difference between sample averages.

Figure 3.6


A
PSR-GFP-PH1
PM
B
sjl1Δ sjl2ts sjl3Δ cells:
26°C
38°C
+ vector
+ 2μ OPY1
+ 2μ PH1OPY1
+ CEN PSR-PH1OPY1
Cell density

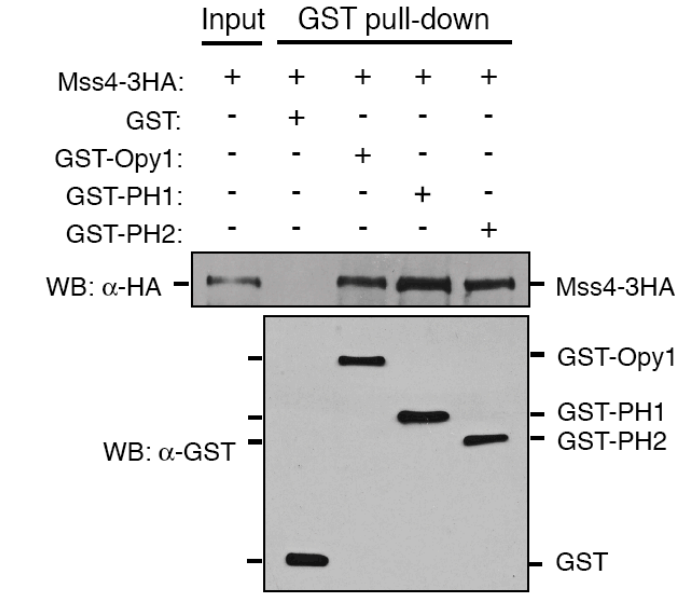

C
Input
GST pull-down
Mss4-3HA: + + + + +
GST: - + - - -
GST-Opy1: - - + - -
GST-PH1: - - - + -
GST-PH2: - - - - +
WB: α-HA
Mss4-3HA
WB: α-GST
GST-Opy1
GST-PH1
GST-PH2
GST

Figure 3.6 PM-targeted Opy1 PH1 domain down-regulates PI(4,5)P_2 levels

(A) Localization of Psr-GFP-PH1 fusion. Scale bar, 5 μm.

(B) PM targeted Opy1 PH1 domain partially rescues the growth defect of *sjl1*Δ *sjl2*^{ts} *sjl3*Δ cells at 38 °C. Serial dilutions of *sjl1*Δ *sjl2*^{ts} *sjl3*Δ cells carrying empty vector or plasmids encoding Opy1, Opy1 PH1 domain, Psr-GFP-PH1 domain as indicated were spotted onto –Ura plates to retain the plasmids and grown at either 26°C or 38°C for 4 days.

(C) GST-Opy1, GST-PH1 and GST-PH2 bind to Mss4-3HA from yeast cell lysates. GST alone does not bind to Mss4-3HA.

Discussion

Using independent proteomic and genetic approaches, we identified the tandem PH domain protein Opy1 as a regulator of Mss4 PIP kinase activity. Our results show that Opy1 has dual functions as a $PI(4,5)P_2$ sensor and regulator. First, localization of Opy1 to cortical patches is dependent on $PI(4,5)P_2$ as well as the N-terminus of Mss4. In addition, over-expression or deletion of Opy1 results in mis-regulation of $PI(4,5)P_2$ synthesis at the PM. Full-length Opy1 protein is mostly cytoplasmic under conditions when $PI(4,5)P_2$ levels at the PM are low (*e.g.* $mss4^{ts}$ cells). Upon activation of $PI(4,5)P_2$ signaling in response to extracellular or intracellular cues, Opy1 may be recruited to Mss4 PIK patches.

We propose that Opy1 down-regulates Mss4 PIP kinase activity at PIK patches through its interaction with the Mss4 N-terminus. Consistent with this model, the N-terminal truncated form of Mss4 displays increased activity compared to full-length Mss4 (Figure 2.5A). Thus, together with PIP 5-phosphatases, Opy1 may be part of a rapid negative regulatory feedback system that is activated as $PI(4,5)P_2$ levels rise. Following attenuation of $PI(4,5)P_2$ synthesis and disassembly of Mss4 PIK patches, Opy1 may then disassociate from the PM (Figure 3.7). Whether Opy1 directly inhibits Mss4 PIP kinase activity or it may act through additional factors is not clear at this point. An *in vitro* PIP kinase assay will likely address this question in the future. It is also interesting to explore what proteins interact with Opy1 in cells to get further details on its function.

Opy1 was originally identified as a protein capable of overcoming mating pheromone induced-G1 arrest in the cell cycle upon over-expression (Edwards et al, 1997). Intriguingly, $PI(4,5)P_2$ is highly concentrated at the shmoo tip in cells responding to pheromone. Maintenance of this anisotropic distribution of $PI(4,5)P_2$ could be necessary for the membrane anchoring and function of downstream signaling complexes (Garrenton et al, 2010). Our results showed that over-expression of Opy1 down-regulates $PI(4,5)P_2$ synthesis at the PM. This may possibly lead to the depletion of this anisotropic $PI(4,5)P_2$ pool that may play a role in cell cycle arrest. Our results give a possible explanation why Opy1 over-expression could override the pheromone induced-cell cycle arrest observed by Edwards and colleagues.

Figure 3.7

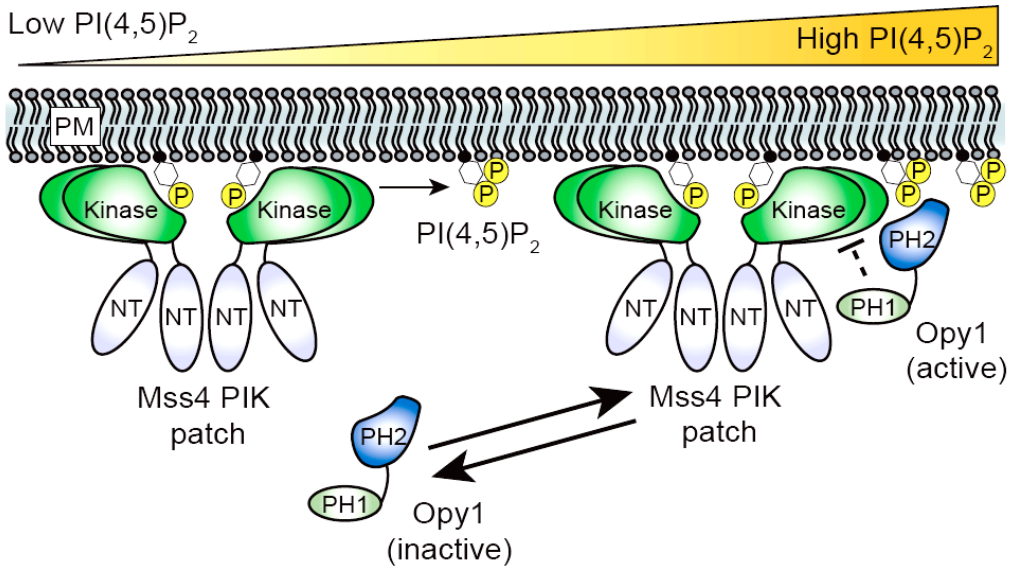

Low PI(4,5)P2
High PI(4,5)P2
PM
Kinase
P
Kinase
PI(4,5)P2
NT
NT
NT
NT
Mss4 PIK patch
PH2
PH1
Opy1 (inactive)
Kinase
Kinase
PH2
PH1
Opy1 (active)
Mss4 PIK patch

Figure 3.7 Model for Opy1 function

Opy1 is in an inactive form when $PI(4,5)P_2$ levels are low. Upon binding $PI(4,5)P_2$, Opy1 is activated and recruited to Mss4 PIK patches in a negative regulatory feedback loop to shut down Mss4 PIP kinase activity. Opy1 may inhibit Mss4 PIP 5-kinase directly or indirectly through an unknown effector (indicated by the dashed line).

References:

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Smaczynska-de R, Il, Costa R, Ayscough KR (2008) Yeast Arf3p modulates plasma membrane PtdIns(4,5)P2 levels to facilitate endocytosis. *Traffic* **9**(4)**:** 559-573

Stefan CJ, Audhya A, Emr SD (2002) The yeast synaptojanin-like proteins control the cellular distribution of phosphatidylinositol (4,5)-bisphosphate. *Mol Biol Cell* **13**(2)**:** 542-557

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Chapter 4

Conclusions and Future Directions[3]

$PI(4,5)P_2$ is essential for a variety of cellular processes, and thus its distribution and abundance must be tightly controlled (Stefan et al, 2002). In this thesis, I demonstrate that the PI4P 5-kinase Mss4 assembles into dynamic, oligomeric structures: PIK patches. The dynamic assembly and disassembly of Mss4 PIK patches may serve as a mechanism for localized synthesis of $PI(4,5)P_2$ at the PM. Oligomerization of Mss4 may provide a platform to precisely modulate PIP kinase activity, as needed, for localized increases in $PI(4,5)P_2$ synthesis through the recruitment of specific activators such as small GTPases (Smaczynska-de et al, 2008), protein kinases/phosphatases (Lee et al, 2005; Ling et al, 2003) and the clathrin coat machinery (Krauss et al, 2006; Nakano-Kobayashi et al, 2007; Sun et al, 2007). In addition, we discovered that the tandem PH domain-containing protein, Opy1, associates with Mss4 at PIK patches and inhibits $PI(4,5)P_2$ synthesis. Thus, Mss4 PIP 5-kinase activity could be attenuated by negative regulatory factors, such as Opy1, prior to the disassembly of PIK patches.

The assembly of Mss4 PIK patches involves the activation loop in the Mss4 lipid kinase domain and its substrate PI4P at the PM. We speculate that

[3] Some of the experiments presented in this chapter were published in Ling Y. *et al*, *EMBO J*, 2012. The dissertation author was the primary investigator and author of the paper.

the conversion of PI4P to PI(4,5)P_2 by Mss4 may lead to PIK patch disassembly as PI4P becomes locally depleted (Figure 2.9). This elegant mechanism would provide an intrinsic switch for the localized control of Mss4 assembly and disassembly reactions at the PM. An electrostatic switch mechanism has been proposed for the membrane association of a mammalian PIP 5-kinase during phagocytosis (Fairn et al, 2009). However, this process involves PIP turnover (by lipases and phosphatases) rather than inherent PIP kinase activity.

Search for additional factors required for Mss4 PIK patch assembly

In vitro lipid-protein binding experiments have revealed that Mss4 binds to both PI4P and PI3P strongly (Figure 2.4D). PI3P mainly localizes to early endosomes and PI4P is also enriched in the Golgi apparatus in addition to the PM (Audhya et al, 2000; Stefan et al, 2002), therefore we would expect Mss4 to localize to these organelles if the lipid-protein interaction is the sole determinant of Mss4 localization. In addition, elevation of PI4P levels (*e.g.* in mutant cells deficient in Sac1 PI4P phosphatase activity) does not result in increased PI(4,5)P_2 levels (Foti et al, 2001). Thus, there must be factors in addition to PI4P that specify the PM localization and the lipid kinase activity of Mss4.

Mis-localization of Mss4 leads to cell death due to low PI(4,5)P_2 levels. We reasoned that the protein(s) that interacts with Mss4 and recruits it to the PM should be essential. To identify upstream proteins required for Mss4 PIK patch

assembly, I carried out a visual screen to examine the localization of Mss4 using the Tet-promoter Hughes yeast collection (Thermo Fisher Scientific). In this collection of ~800 yeast strains, the endogenous promoters of essential genes are replaced by a doxycycline sensitive promoter (Figure 4.1A). The advantage of using this strain collection is that the expression of an essential gene product could be switched off by adding doxycycline to the growth medium. Irrelevant genes, such as those encoding transcription factors and mitochondria proteins, were not tested in my study. By visualizing Mss4-GFP localization in cells that are grown in the presence of 50 μg/mL doxycycline, I identified 13 candidate genes that are important for Mss4 PM localization (Figure 4.1B and Table 3), which I term *CML1-13* (***C***ontrol ***M***ss4 ***L***ocalization). These *CML* gene products control a variety of cellular processes. Two Cml proteins are components of the Arp2/3 complex (e.g. Arc35 and Arp2); other Cml proteins regulate important cellular pathways including membrane trafficking, lipid metabolism, protein modification and protein folding (Table 3).

The depolymerization of actin filaments does not affect Mss4 PIK patch assembly at the PM (Figure 2.2A). Therefore the two essential components of the Arp2/3 complex, Arc35 and Arp2, may play additional roles in affecting Mss4 PM targeting in cells. Mss4 undergoes nuclear-cytoplasmic shuttling (Audhya & Emr, 2003), and these Cml proteins may indirectly control Mss4 PIK patch assembly at the PM by affecting this trafficking process. The functions of several Cml proteins also suggest they may regulate the localization of Mss4 by

modulating the lipid composition of the membranes. The discovery of these Cml proteins implicates many distinct pathways in the localization of Mss4. Further studies are needed to determine the *in vivo* localization of these Cml proteins and examine if they directly interact with Mss4. Another possibility is that Mss4 is recruited to the PM by a number of different activators. These activators may not have essential functions alone, but disruption of several of these proteins may lead to defects in $PI(4,5)P_2$ synthesis and cell growth. Therefore, another approach, likely an optimized mass spectrometry experiment for trans-membrane protein identification, is needed to discover what proteins can directly recruit Mss4 to the PM in the future.

Conserved roles of dual PH domain proteins in regulating PIP signaling

Using independent proteomic and genetic approaches, we identified the tandem PH domain protein Opy1 as a $PI(4,5)P_2$ sensor and regulator of Mss4 PIP kinase activity. Intriguingly, mammalian cells have several putative Opy1 homologues with tandem PH domains (Figure 4.2A). Three members of this protein family, pleckstrin, TAPP1, and TAPP2, bind $PI(3,4)P_2$ through their PH2 domains (Allam & Marshall, 2005; Dowler et al, 2000; Edlich et al, 2005; Haslam et al, 1993). In addition, TAPP1, TAPP2, and pleckstrin were reported to inhibit PI3K signaling (Abrams et al, 1996; Wullschleger et al, 2011).

Figure 4.1

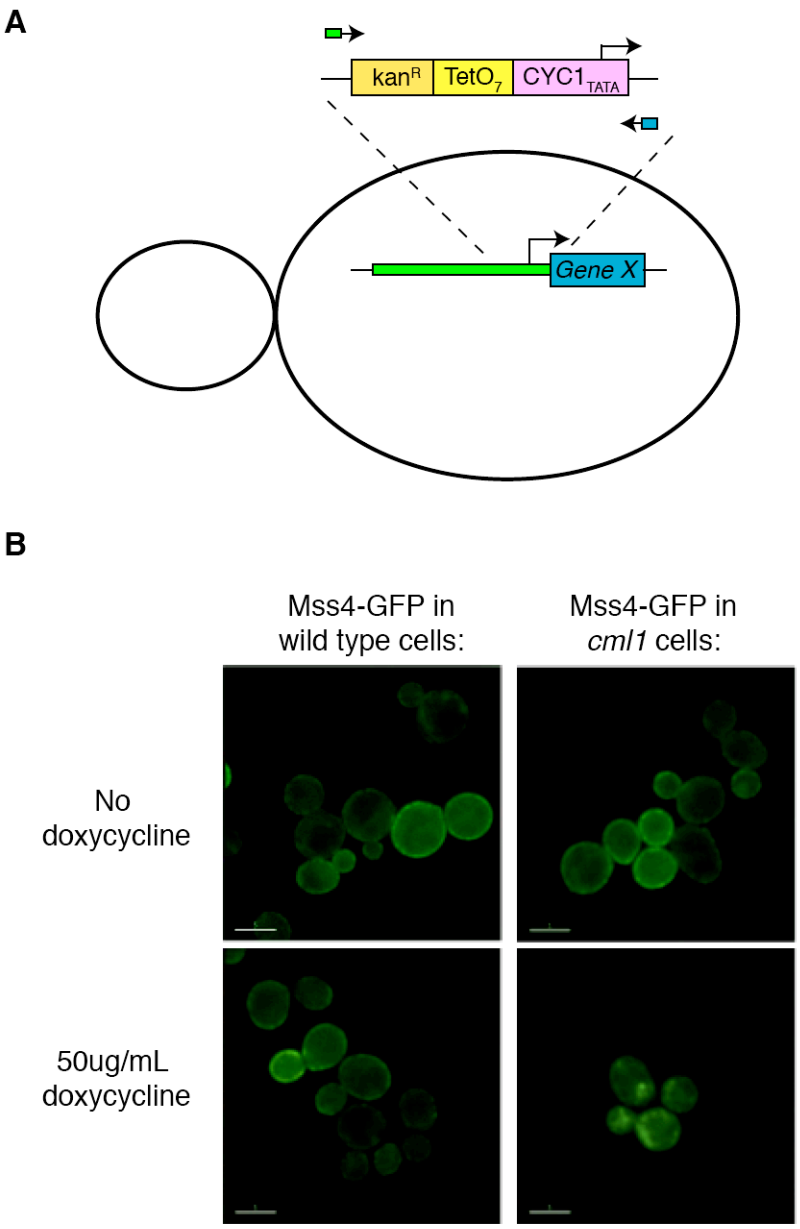
A
kanR
TetO7
CYC1TATA
Gene X
B
Mss4-GFP in wild type cells:
Mss4-GFP in cml1 cells:
No doxycycline
50ug/mL doxycycline

Figure 4.1 Visual screens to identify proteins required for Mss4 PIK patch assembly

(A) Schematic diagrams of the Tet-promoter Hughes yeast collection. The endogenous promoter of an essential gene X is replaced by the Tet-promoter. By adding doxycycline to the growth media, the transcription of the essential gene can shut off.

(B) Identification of *CML* (Control Mss4 Localization) genes. The localization of Mss4-GFP was examined in the selected strains either in the presence or the absence of doxycycline. The results for the *CML1* gene (*ARC35*) are shown. Scale bar, 5 μm. Details for experiments are described in Chapter 3.

Table 3. Genes required for Mss4 PIK patch assembly		
Name	**Gene**	**Gene product function**
CML1	*ARC35*	Subunit of the ARP2/3 complex, which is required for the motility and integrity of cortical actin patches; required for cortical localization of calmodulin.
CML2	*ARP2*	Essential component of the Arp2/3 complex, which is a highly conserved actin nucleation center required for the motility and integrity of actin patches; involved in endocytosis and membrane growth and polarity.
CML3	*DSL1*	Peripheral membrane protein needed for Golgi-to-ER retrograde traffic; forms a complex with Sec39p and Tip20p that interacts with ER SNAREs Sec20p and Use1p; component of the ER target site that interacts with coatomer; interacts with Cin5p.
CML4	*ERG29*	Protein of unknown function that may be involved in iron metabolism; shows localization to the ER; highly conserved in ascomycetes.
CML5	*GAB1*	GPI transamidase subunit, involved in attachment of glycosylphosphatidylinositol (GPI) anchors to proteins; may have a role in recognition of the attachment signal or of the lipid portion of GPI.
CML6	*PHS1*	Essential 3-hydroxyacyl-CoA dehydratase of the ER membrane, involved in elongation of very long-chain fatty acids; evolutionarily conserved, similar to mammalian PTPLA and PTPLB; involved in sphingolipid biosynthesis and protein trafficking.
CML7	*PMA1*	Plasma membrane H+-ATPase, pumps protons out of the cell; major regulator of cytoplasmic pH and plasma membrane potential; P2-type ATPase.
CML8	*RFT1*	Essential integral membrane protein that is required for translocation of Man5GlcNac2-PP-Dol from the cytoplasmic side to the lumenal side of the ER membrane but is not the flippase; mutation is suppressed by expression of human p53 protein.
CML9	*ROT1*	Molecular chaperone involved in protein folding in the ER; mutation causes defects in cell wall synthesis and in lysis of autophagic bodies, suppresses tor2 mutations, and is synthetically lethal with kar2-1 and with rot2 mutations.
CML10	*SAR1*	GTPase, GTP-binding protein of the ARF family, component of COPII coat of vesicles; required for transport vesicle formation during ER to Golgi protein transport.
CML11	*SEC14*	Phosphatidylinositol/phosphatidylcholine transfer protein; involved in regulating PtdIns, PtdCho, and ceramide metabolism, products of which regulate intracellular transport and UPR; functionally homologous to mammalian PITPs.
CML12	*SEC21*	Gamma subunit of coatomer, a heptameric protein complex that together with Arf1p forms the COPI coat; involved in ER to Golgi transport of selective cargo.
CML13	*YJR141w*	Uncharacterized.

To test whether these proteins have conserved functions in regulating PI(4,5)P_2 synthesis, I over-expressed pleckstrin or TAPP1 in *sjl1*Δ *sjl2*^{ts} *sjl3*Δ cells that accumulate toxic levels of PI(4,5)P_2. Interestingly, TAPP1, but not pleckstrin, weakly rescued the growth defect of *sjl1*Δ *sjl2*^{ts} *sjl3*Δ cells at the non-permissive temperature (Figure 4.2B). And consistently, PI(4,5)P_2 levels were restored to normal in *sjl1*Δ *sjl2*^{ts} *sjl3*Δ cells upon over-expression of TAPP1 (Figure 4.2C). To address whether inhibition of PI(4,5)P_2 synthesis by TAPP1 occurred through Mss4-TAPP1 interactions, cell lysates containing GFP-TAPP1 were incubated with His$_6$-SUMO-Mss4$^{1-346}$ or His$_6$-SUMO-Mss4$^{347-779}$ fusion proteins bound to nickel-charged agarose beads. Interestingly, both His$_6$-SUMO-Mss4$^{1-346}$ and His$_6$-SUMO-Mss4$^{347-779}$ fusion proteins pulled down GFP-TAPP1 from cell lysates, but not GFP alone (Figure 4.2D).

The inhibition of PI3Kγ activity by pleckstrin has been proposed to occur by direct interaction between PI3Kγ and pleckstrin, or alternatively through binding of pleckstrin to PI3Kγ activators, the βγ subunits of heterotrimeric GTP-binding proteins (Abrams et al, 1996). TAPP1 and TAPP2 also down-regulate insulin and PI(3,4,5)P_3 signaling upon generation of PI(3,4)P_2 by the PIP 5-phosphatase SHIP2 (Wullschleger et al, 2011). TAPP1 and TAPP2 may regulate PI(3,4,5)P_3 levels in cells by mechanisms analogous to pleckstrin. Our results suggest yet another possible model for the control of PI3K signaling by dual PH domain proteins–inhibition of PIP 5-kinases and PI(4,5)P_2 synthesis that would impinge on PI3K activity (Figure 4.3B).

Figure 4.2

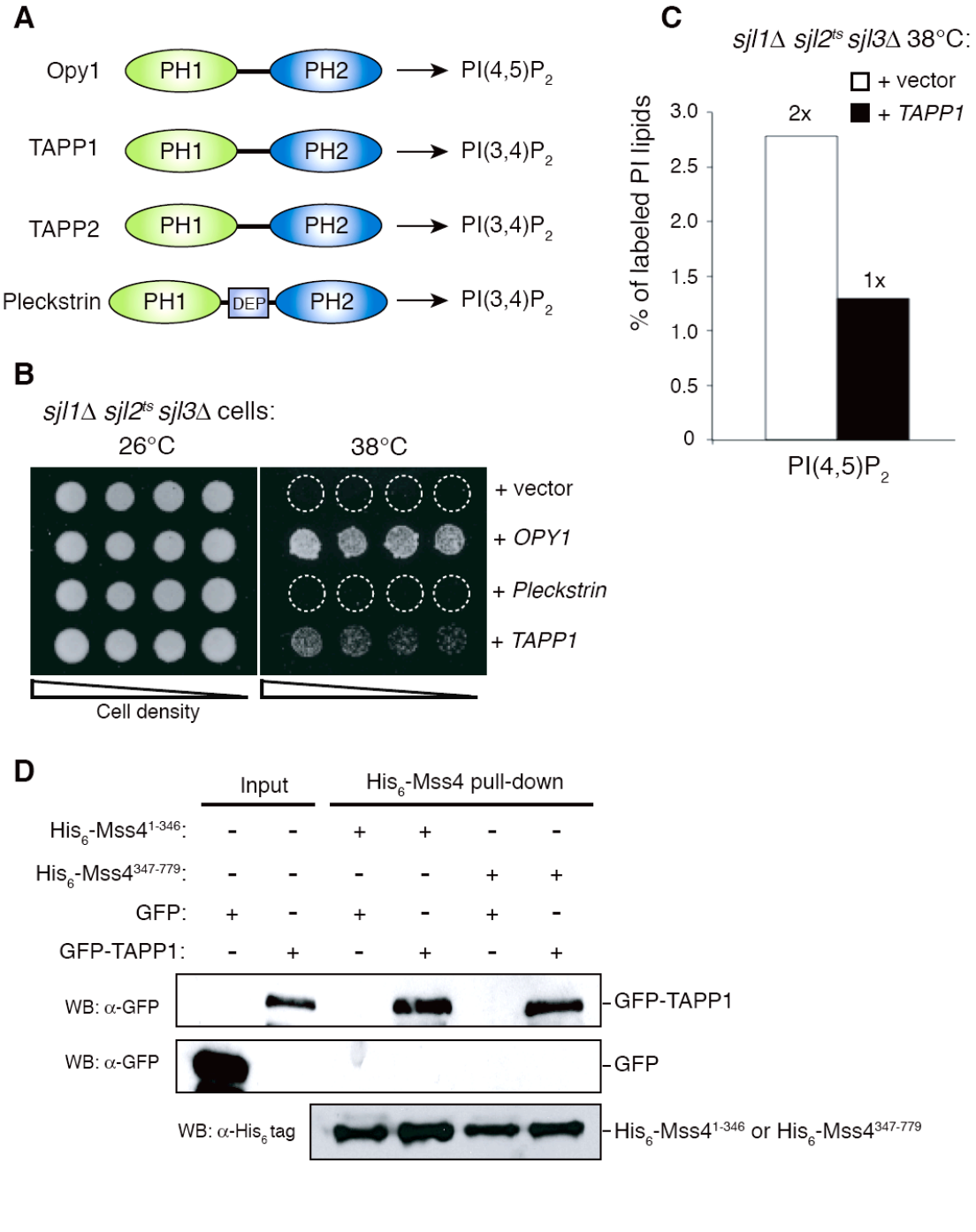
A
Opy1
PH1
PH2
PI(4,5)P2
TAPP1
PH1
PH2
PI(3,4)P2
TAPP2
PH1
PH2
PI(3,4)P2
Pleckstrin
PH1
DEP
PH2
PI(3,4)P2
B
sjl1Δ sjl2ts sjl3Δ cells:
26°C
38°C
+ vector
+ OPY1
+ Pleckstrin
+ TAPP1
Cell density
C
sjl1Δ sjl2ts sjl3Δ 38°C:
+ vector
+ TAPP1
% of labeled PI lipids
3.0
2.5
2.0
1.5
1.0
0.5
0
2x
1x
PI(4,5)P2
D
Input
His6-Mss4 pull-down
His6-Mss41-346:
His6-Mss4347-779:
GFP:
GFP-TAPP1:
WB: α-GFP
GFP-TAPP1
WB: α-GFP
GFP
WB: α-His6 tag
His6-Mss41-346 or His6-Mss4347-779

Figure 4.2 The Opy1 homolog, TAPP1, rescues the growth defects of yeast synaptojanin mutant cells

(A) Schematic diagrams of the Opy1, TAPP1, TAPP2, and pleckstrin tandem PH domain proteins are shown. PH, pleckstrin homology domain; DEP, Disheveled/EGL-10/Pleckstrin homology domain.

(B) Over-expression of full-length Opy1 or TAPP1, but not pleckstrin, rescues the growth defect of *sjl1*Δ *sjl2*^{ts} *sjl3*Δ cells at 38°C. Serial dilutions of *sjl1*Δ *sjl2*^{ts} *sjl3*Δ cells carrying empty vector or plasmids encoding Opy1, TAPP1, or pleckstrin as indicated were spotted onto –Ura plates to retain the plasmids and grown at either 26°C or 38°C for 4 days.

(C) Cellular PI(4,5)P_2 levels as measured by ^{3}H-inositiol labeling and HPLC analysis in *sjl1*Δ *sjl2*^{ts} *sjl3*Δ cells carrying empty vector or *TAPP1* plasmids. Two independent labeling experiments were performed at 38°C. Results from a representative experiment are shown. Additional data are provided in Table 2.

(D) His_6-SUMO-Mss4$^{1-346}$ and His_6-SUMO-Mss4$^{347-779}$ bind to GFP-TAPP1 from yeast cell lysates. GFP alone does not bind to His_6-SUMO-Mss4$^{1-346}$ and His_6-SUMO-Mss4$^{347-779}$. Inputs for His_6-SUMO-Mss4$^{1-346}$ and His_6-SUMO-Mss4$^{347-779}$ for each binding reaction are shown (bottom).

Though the PIP binding specificities of pleckstrin, TAPP1, TAPP2, and Opy1 have diverged, they share similar roles in attenuating PIP signaling. We propose that the C-terminal PH2 domains of these proteins serve as PIP sensors (and possibly PIP kinase interactor), while the N-terminal PH1 domains regulate PIP kinases. Consistent with this, PH domains are involved in protein-protein interactions in addition to PIP binding (Wang et al, 1994; Yao et al, 1994). It remains unclear whether these tandem PH domain proteins directly inhibit PIP kinases or indirectly act through additional factors. Opy1 was originally identified as a regulator of GPCR and MAPK signaling in yeast (Edwards et al, 1997) and MAPK activation in yeast requires localized $PI(4,5)P_2$ signaling (Garrenton et al, 2010). Our study links the regulation of GPCR, MAPK, and PIP signaling through Opy1 function. It is therefore interesting that pleckstrin and TAPP1, TAPP2 control PI3K activity in response to GPCR and insulin receptor signaling. Thus, future studies on this interesting family of dual PH domain proteins will likely provide new insight into our understanding of PIP signaling pathways. It is also important to further study how the oligomerization/assembly of PIP kinases is regulated at cellular membranes and how PIP kinase activity is precisely controlled by upstream signals in the future.

Figure 4.3

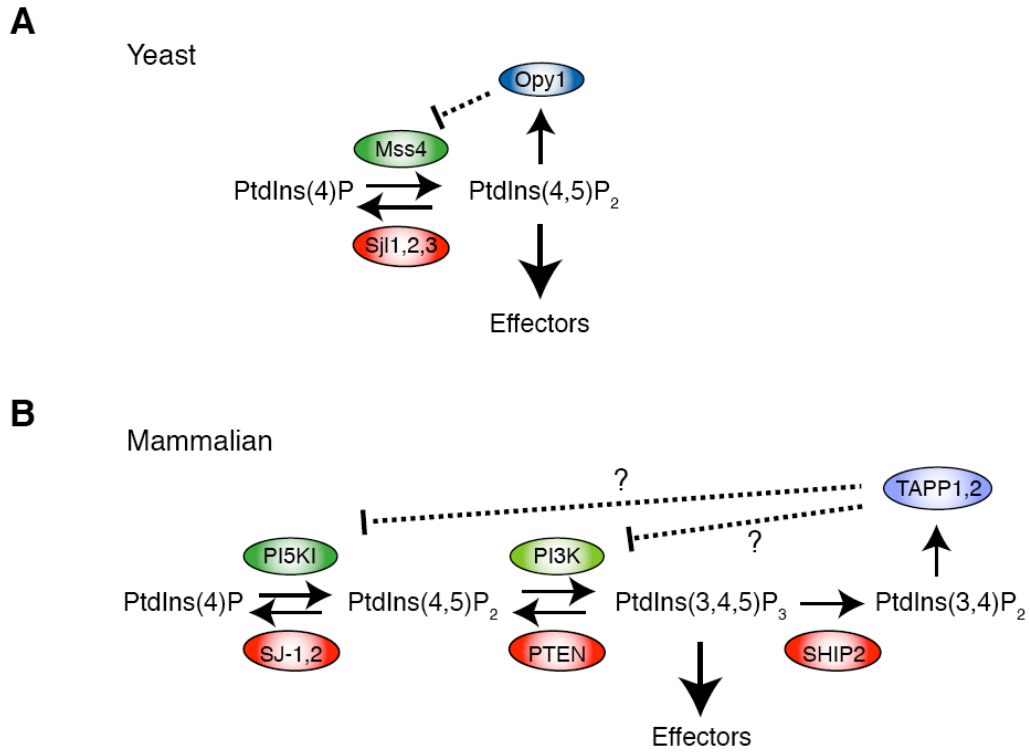
A
Yeast
Opy1
Mss4
PtdIns(4)P
PtdIns(4,5)P2
Sjl1,2,3
Effectors
B
Mammalian
?
TAPP1,2
?
PI5KI
PI3K
PtdIns(4)P
PtdIns(4,5)P2
PtdIns(3,4,5)P3
PtdIns(3,4)P2
SJ-1,2
PTEN
SHIP2
Effectors

Figure 4.3 Model for Opy1 and TAPP1/TAPP2 functions

(A) Model for Opy1 function in yeast cells.

(B) Model for TAPP1, TAPP2 function in mammalian cells.

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Smaczynska-de R, II, Costa R, Ayscough KR (2008) Yeast Arf3p modulates plasma membrane PtdIns(4,5)P2 levels to facilitate endocytosis. *Traffic* **9**(4)**:** 559-573

Stefan CJ, Audhya A, Emr SD (2002) The yeast synaptojanin-like proteins control the cellular distribution of phosphatidylinositol (4,5)-bisphosphate. *Mol Biol Cell* **13**(2)**:** 542-557

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Table S1. Strains used in this study.		
Strain	**Genotype**	**Reference**
SEY6210	*MATa leu2-3,112 ura3-52 his3Δ200 trp1-Δ901 lys2-801 suc2Δ9*	(Robinson et al, 1988)
SEY6210.1	*MAT**a** leu2-3,112 ura3-52 his3Δ200 trp1-Δ901 lys2-801 suc2Δ9*	(Robinson et al, 1988)
AAY202	SEY6210 *mss4Δ::HIS3* and harboring YCp*lac*111*mss4^{ts}-102*	(Stefan et al, 2002)
AAY1207	SEY6210 *MSS4-GFP::HIS3MX6*	(Audhya & Emr, 2003)
YYL023	6210.5 *MSS4-3HA:: HIS3MX6*	This study
YYL044	6210.5 *MSS4-13myc::TRP1*	This study
YYL045	6210.5 *MSS4-3HA:: HIS3MX6 MSS4-13myc::TRP1*	This study
YWL980	*MATa leu2-Δ1 ura3-52 his3Δ200 trp1-Δ63 lys2-801 CSE4-3xGFP::TRP1*	(Markus et al, 2009)
YYL102	YML980 *MSS4-GFP::HIS3MX6*	This study
AAY201	SEY6210 *mss4Δ::HIS3* harboring pRS416*MSS4*	(Stefan et al, 2002)
YYL111	SEY6210 *mss4Δ::HIS3* harboring pRS414*MSS4-GFP*	This study
YCS396	SEY6210 *pil1Δ::HIS3*	(Karotki et al, 2011)
YCS429	SEY6210 *PIL1-mCherry::HIS3MX6*	(Karotki et al, 2011)
YCS337	SEY6210 *ABP1-mRFP:: HIS3MX6*	(Stefan et al, 2005)
AAY102	SEY6210 *stt4 Δ ::HIS3* harboring pRS415*stt4-4*	(Audhya et al, 2000)
AAY104	SEY6210 *pik1 Δ ::HIS3* harboring pRS314*pik1-83*	(Audhya et al, 2000)
AAY105	SEY6210 *stt4Δ::HIS3 pik1Δ::HIS3* harboring pRS415*stt4-4* and pRS314*pik1-83*	(Audhya et al, 2000)
JMY566	SEY6210 *arg4Δ::kan^{r}*	This study
YYL154	JMY566 *MSS4-3xFLAG::HIS3MX6*	This study
YYL134	SEY6210 *MSS4-3xFLAG::HIS3MX6*	This study
YYL161	SEY6210 *OPY1-GFP::HIS3MX6*	This study
YYL162	SEY6210 *OPY1-3HA::TRP1*	This study
YYL163	SEY6210 *MSS4-FLAG::HIS3MX6 OPY1-3HA::TRP1*	This study
AAY1940	SEY6210 *opy1Δ::HIS3*	This study
YCS176	SEY6210 *sjl1Δ::hisG sjl2Δ::HIS3 sjl3Δ::TRP1* harboring pRS415*sjl2^{ts}-8*	(Stefan et al, 2002)
YCS189	SEY6210 *sjl1Δ::hisG sjl2Δ::HIS3 sjl3Δ::TRP1 inp54Δ* harboring pRS415*sjl2^{ts}-8*	This study
YYL169	SEY6210.1 *OPY1-GFP::HIS3MX6 mss4Δ::HIS3* harboring pRS414*MSS4-RFPmars*	This study
YYL170	SEY6210.1 *OPY1-GFP::HIS3MX6 mss4Δ::HIS3* harboring pRS414*MSS4-3HA*	This study
YYL171	SEY6210.1 *OPY1-GFP::HIS3MX6 mss4Δ::HIS3* harboring pRS414*MSS4Δ2-346 -3HA*	This study
YCS239	SEY6210.1 *sjl1Δ::HIS3 sjl2Δ::HIS3*	(Garrenton et al, 2010)
YYL174	SEY6210 *sjl1Δ::HIS3 sjl2Δ::HIS3 opy1Δ::HIS3* harboring pRS426 *SJL2-GFP*	This study
YTS1	SEY6210.1 *ymr1Δ::HIS3 sjl2Δ::HIS3 sjl3Δ::TRP1* harboring pRS415*ymr1ts*	(Parrish et al, 2005)
AAY143b	SEY6210 *sjl2Δ::HIS3 sjl3Δ::TRP1 sac1Δ::TRP1* harboring pRS415*sac1-23*	(Foti et al, 2001)
MFY62	SEY6210.1 *sac1Δ::TRP1*	(Foti et al, 2001)
YYL168	SEY6210 *opy1Δ::HIS3 sac1Δ::TRP1*	This study
YYL153	*yck1Δ yck2-2 arg4Δ::KanMx MSS4-3xFLAG::HIS3 leu2 ura3 bar1*	This study

Table S2. Plasmids used in this study.		
Plasmid	**Description**	**Source**
pCS276	pRS416-*MSS4-GFP*	This study
pYL105	pRS414-*MSS4-GFP*	This study
pYL203	pRS414-*MSS4*$^{\Delta 2\text{-}346}$*-GFP*	This study
pYL172	pRS414-*MSS4*$^{\Delta 347\text{-}364}$*-GFP*	This study
pYL204	pRS414-*MSS4*$^{\Delta 726\text{-}779}$ *-GFP*	This study
pYL143	pRS414- *MSS4* ^{KK720,721DD}*-GFP*	This study
pYL142	pRS414- *MSS4* ^{KK720,721RR}*-GFP*	This study
pYL211	pRS414- *MSS4* ^{T312A}*-GFP*	This study
pYL212	pRS414- *MSS4* ^{T312D}*-GFP*	This study
pYL147	pRS414- *MSS4* ^{S477A}*-GFP*	This study
pYL148	pRS414- *MSS4* ^{S477D}*-GFP*	This study
pYL080	pRS416- *MSS4* ^{S728A}*-GFP*	This study
pYL081	pRS416- *MSS4* ^{S728D}*-GFP*	This study
pYL109	pRS414-*MSS4-3xHA*	This study
pYL137	pRS414-*MSS4*$^{\Delta 2\text{-}346}$*-3xHA*	This study
pCS111	pRS416-*SJL2*	(Stefan et al, 2002)
pYL178	Yep352-*OPY1*	This study
pYL183	Yep352-PH1^{OPY1}	This study
pYL179	Yep352-PH2^{OPY1}	This study
pYL174	pRS416-*OPY1*-GFP	This study
pYL205	pRS416-PH1^{OPY1}-GFP	This study
pYL177	pRS416-PH2^{OPY1}-GFP	This study
pYL189	pRS416-pCPY1-PSR-GFP-PH1^{OPY1}	This study
pYL210	pRS416-pGPD1-GFP-*OPY1*	This study
pYL206	pRS416-pGPD1-GFP-*pleckstrin*	This study
pYL207	pRS416-pGPD1-GFP-*TAPP1*	This study
ppSUMO	pET28a based His_6-SUMO vector	Sondermann Lab, Cornell University
pYL194	ppSUMO-*MSS4*$^{1\text{-}346}$	This study
pYL159	ppSUMO-*MSS4*$^{347\text{-}779}$	This study
pYL160	ppSUMO-*MSS4*$^{454\text{-}779}$	This study
pYL168	pRS416-pGPD1-GFP-YCK1	This study
pYL169	pRS416-pGPD1-GFP-YCK2	This study